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14. ABSTRACT

The ingestion of food antigens plays an essential role in the development of eosinophilic esophagitis (EE) as total removal of dietary antigens by using an amino acid based oral formula improves clinical symptoms and esophageal histology in 98% of patients with EE within a month. EE is thought to be mediated by both IgE and non-IgE mediated food allergy. In this study we are particularly interested in identifying genes in EE linked to a significant complication of EE namely esophageal stricture formation. This study focuses on increasing our understanding of two genes (TGF-b and acidic chitinase) associated with remodeling and stricture formation in the esophagus in EE. The importance of TGF-b and acidic chitinase to the development of egg induced remodeling of the esophagus is being studied in a mouse model of egg induced EE in which either TGF-b signaling or acidic chitinase activity is neutralized. Our studies demonstrate that targeting Smad3 or chitinase both partially reduced egg induced esophageal fibrosis associated with EE. The combination of targeting Smad3 and chitinase may be more effective than targeting each pathway alone.

15. SUBJECT TERMS
Eosinophils, smad-3, chitinase, remodeling, food allergy

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Introduction:
The ingestion of food antigens plays an essential role in the development of eosinophilic esophagitis (EE) as total removal of dietary antigens by using an amino acid based oral formula improves clinical symptoms and esophageal histology in 98% of patients with EE within a month. EE is thought to be mediated by both IgE and non-IgE mediated food allergy. In this study we are particularly interested in identifying genes in EE linked to a significant complication of EE namely esophageal stricture formation. This study focuses on increasing our understanding of two genes namely a) TGF-b (transforming growth factor-b), and b) acidic chitinase, to determine their role in remodeling and stricture formation in the esophagus in EE. The importance of TGF-b and acidic chitinase to the development of egg induced remodeling of the esophagus is being studied in a mouse model in which either TGF-b signaling is inhibited or acidic chitinase activity is neutralized. TGF-b will be inactivated in studies of Smad3 deficient mice (essential for TGF-b signaling) and chitinase will be inactivated in studies of mice administered allosamidin a pharmacologic inhibitor of chitinase.

Body:
This proposal outlines 4 tasks to be completed during the three year proposal. We have completed Task 1-3 and submitted original manuscripts (Task 4) as planned in our original proposal.

1. **Task 1:** Breeding of Smad3 deficient mice (month 1 to 3)
We have completed task 1 the breeding of Smad 3 deficient mice and used them in experiments proposed for task 2.

2. **Task 2:** Mouse model of egg induced EE (WT vs Smad3 deficient) (month 4 to 16)
We have completed all the task 2 experiments proposed:
Task 2 (a) Exposure of WT and Smad3 deficient mice to OVA allergen
Task 2 (b) Quantitating fibrosis
Task 2 (c) Quantitating basal zone hyperplasia
Task 2 (d) Quantitating blood vessels
Task 2 (e) Quantitating eosinophils
Task 2 (f) Quantitating TGF-b+ cells
Task 2 (g) Quantitating pSmad+ cells

3. **Task 3:** Mouse model of egg induced EE (WT vs WT + allasomidin a pharmacologic inhibitor of chitinase) (month 16 to 28)
We have completed the task 3 experiments proposed:
Task 3 (a) Exposure of WT and WT + allasomidin to OVA allergen
Task 3 (b) Quantitating fibrosis
Task 3 (c) Quantitating basal zone hyperplasia
Task 3 (d) Quantitating blood vessels
Task 3 (e) Quantitating eosinophils
Task 3 (f) Quantitating TGF-b+ cells
Task 3 (g) Quantitating pSmad+ cells
Task 3 (h) Quantitation of chitinase + cells

4. **Task 4:** Preparation of manuscript (month 33 to 36)
We have submitted the following two original manuscripts (see both manuscripts in appendix; one published, one revised submission) describing our findings related to task 2 and task 3 :

- Jae Youn Cho, Peter Rosenthal, Marina Miller, Alexa Pham, Seema Aceves, Shohei Sakuda, and **David H Broide.** Targeting AMCase reduces esophageal eosinophilic inflammation and remodeling in a mouse model of egg induced eosinophilic esophagitis. *Int Immunopharmacology* (2014) 18: 35-42.

- JaeYoun Cho, Ashmi Doshi, Peter Rosenthal, Andrew Beppu, Marina Miller, Seema Aceves, **David Broide.** Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis. *Journal of Pediatric Gastroenterology & Nutrition*. (2014). Revised manuscript submitted (see decision letter below).

From: JPGN North America <emily.senerth@wolterskluwer.com>
To: David Broide <dbroide@ucsd.edu>
Date: 25 Apr 2013
Subject: JPGN-NA Decision

RE: JPGN-NA-13-136; "Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis"

Dear Dr. Broide,

I am pleased to inform you that the above-referenced manuscript has been found potentially acceptable for publication. However, a final decision cannot be made until there has been significant revision to address the concerns of the reviewers. The reviewers' comments are provided below.

Kind Regards,

Melvin Heyman, MD, MPH
Editor-in-Chief
Journal of Pediatric Gastroenterology & Nutrition
http://jpgn-na.edmgr.com/

We have performed additional experiments to address the reviewer comments and submitted a revised manuscript in January 2014.

Key Research Accomplishments:

- **Smad-3 deficient mice have significantly less esophageal fibrosis and angiogenesis in a mouse model of egg induced EE.**

The first aim of the study was to determine whether Smad-3 deficient mice have reduced remodeling of the esophagus in a mouse model of egg (i.e. OVA) induced eosinophilic esophagitis. The studies we performed confirmed this hypothesis as Smad3 KO mice challenged intragastrically with OVA had significantly less esophageal fibrosis (assessed by quantitating the area of esophageal trichrome staining which stains collagen) and angiogenesis compared to WT mice. The key research accomplishments and results of this study are detailed below listed as "Smad 3 manuscript Results Figure 1-6" which are also included in the attached Manuscript in the Appendix (JaeYoun Cho, Ashmi Doshi, Peter Rosenthal, Andrew Beppu, Marina Miller, Seema Aceves, **David Broide.** Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis. *Journal of Pediatric Gastroenterology & Nutrition*. (2014). Revised manuscript submitted).

Smad 3 Manuscript Results Figure 1-6:

MBP+ esophageal eosinophils

The number of esophageal eosinophils increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (122.9 $\pm$ 24.8 vs. 12.2 $\pm$ 2.2 eosinophils/mm^2; p<0.0001)(**Figure 1**). OVA challenge in Smad3 deficient mice induced a similar increase in the number of esophageal eosinophils (OVA Smad3 KO vs no OVA Smad3 KO; p<0.0001) as noted in OVA challenged WT mice (**Figure 1**).

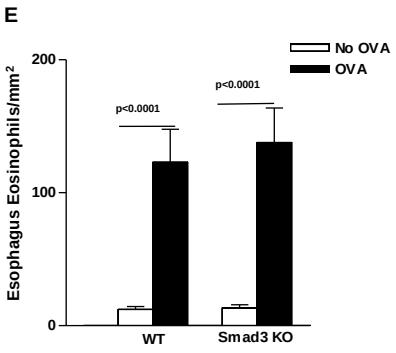


Figure 1

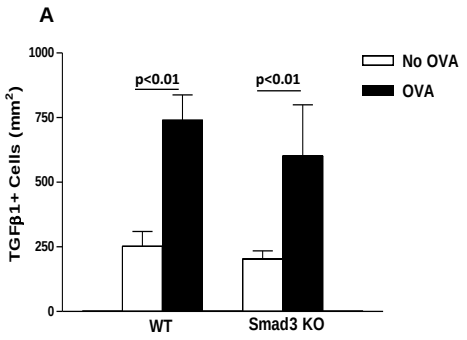


Figure 2A

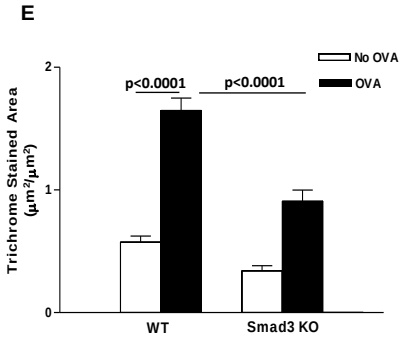


Figure 3

Esophageal IL-5+ cells and eotaxin+ cells
There was no difference in the number of IL-5 positive cells in OVA challenged WT mice compared to OVA challenged Smad3 KO mice (0.72 $\pm$ 0.33 vs 0.85 $\pm$ 0.45 IL-5 positive cells/mm^2; p=ns). Eotaxin-1 positive cells were not detected in either WT or Smad3 KO mice.

Esophageal TGF-β1+ cells and esophageal TGF-β1 mRNA
The number of esophageal TGF-β1+ cells increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (p<0.01)(**Figure 2A**). There was no significant difference in the number of esophageal TGF-β1+ cells in OVA challenged Smad3 deficient mice compared to OVA challenged WT mice (p=ns)(**Figure 2A**). Levels of esophageal TGF-β1 mRNA increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (p<0.01). There was no significant difference in levels of esophageal TGF-β1 mRNA in OVA challenged Smad3 deficient mice compared to OVA challenged WT mice (p=ns).

Esophageal Fibrosis
The area of esophageal trichrome staining (which detects collagen) increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (p<0.0001)(**Figure 3**). Smad3 deficient mice challenged with OVA had a significant reduction in the area of esophageal trichrome staining compared to OVA challenged WT mice (p<0.0001)(**Figure 3**).

Esophageal angiogenesis
WT mice challenged with OVA had a significant increase in the number of esophageal small blood vessels as quantitated by PECAM staining (p<0.0001 vs no OVA)(**Figure 4**). In contrast, OVA challenged Smad3 deficient mice had a significant reduction in the number of esophageal small blood vessels compared to OVA challenged WT mice (p< 0.0001)(**Figure 4**).

Esophageal VEGF positive cells
To determine whether the Smad3 pathway influenced levels of angiogenic cytokines, we quantitated the number of VEGF positive cells within the lamina propria. WT mice challenged with OVA had a significant increase in the number of VEGF positive cells in the LP (p<0.0001; vs WT no OVA)(**Figure 5).** Smad3 deficient mice challenged with OVA had significantly reduced numbers of VEGF positive cells in the LP (p<0.0001; vs WT OVA) (**Figure 5**).

Esophageal Basal Zone Thickness
OVA challenged WT mice had a slight increase in basal zone thickness compared to non-OVA challenged WT mice which was not statistically significant (p=0.12). OVA challenged Smad3 deficient mice had a trend for reduction in basal layer thickness compared to OVA challenged WT mice which was not statistically significant (p=0.40).

Effect of TGF- β1 on Smad3 deficient versus WT mast cell proliferation
WT MBMMC cultured in the presence of TGF-β1 for 48 hours had a significantly greater inhibition of MBMMC proliferation (48% inhibition) compared to Smad3 deficient MBMMC cultured in the presence of TGF-β1 (18 % inhibition) (p <0.001)(**Figure 6).**

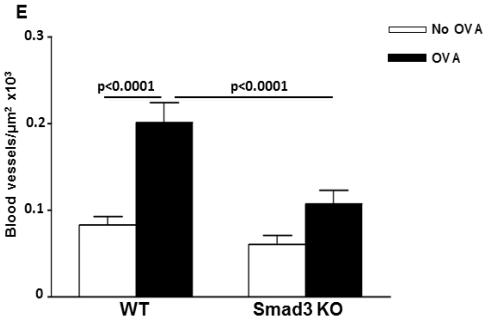


Figure 4

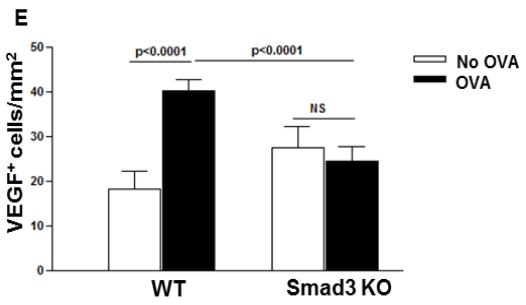


Figure 5

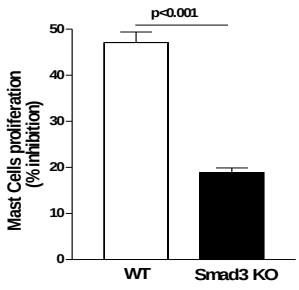


Figure 6

Smad 3 study Key accomplishment conclusion:
As Smad-3 mediates TGF-b signaling these studies suggest that the TGF-b/TGF-b receptor/Smad-3 pathway is activated in inducing esophageal fibrosis. The cellular source of TGF-b in EE are predominantly esophageal eosinophils, whereas the cell that responds to TGF-b released by eosinophils are esophageal fibroblasts which express TGF-b receptors which

activate intracellular Smad-3 signaling pathways to promote fibrosis. Thus, as anticipated the number of esophageal eosinophils and esophageal cells expressing TGF-b was not significantly different in WT and Smad-3 deficient mice challenged intragastrically with OVA. The key difference between WT and Smad3 deficient mice was the inability of Smad3 deficient mouse esophageal fibroblasts to respond to TGF-b by activating the Smad3 pathway. These results suggest that targeting Smad3 with an oral small molecule inhibitor may be a novel way to inhibit esophageal fibrosis and esophageal stricture formation which is a major complication of human EE.

- **Targeting chitinase with allosamidin reduces eosinophilic inflammation and esophageal fibrosis in a mouse model of egg induced EE.**

The second aim of this study investigated whether pharmacologic inhibition of chitinase (using allosamedin) inhibited esophageal eosinophilic inflammation and remodeling in a mouse model of OVA (egg) induced EE. The key research accomplishments and results of this second aim are detailed below listed as "Targeting AMCase manuscript Results Figure 1-6" which are also included in the attached Manuscript in the Appendix (Jae Youn Cho, Peter Rosenthal, Marina Miller, Alexa Pham, Seema Aceves, Shohei Sakuda, and **David Broide.** Targeting AMCase reduces esophageal eosinophilic inflammation and remodeling in a mouse model of egg induced eosinophilic esophagitis. *Int Immunopharmacology* (2014) 18: 35-42).

Targeting AMCase Manuscript Results Figure 1-6:

Allosamidin inhibits oral OVA induced eosinophilic inflammation in the esophagus

Oral OVA challenge induced a significant increase in esophageal eosinophils. Eosinophils were infiltrated into the esophageal epithelium, lamina propria, and muscle layer. OVA challenge increased the total number of MBP+ eosinophils in the esophagus including the epithelium (OVA vs no OVA)(p=0.0001)(**Fig 1C**), the lamina propria (OVA vs no OVA)(p =0.0001)(**Fig 1D**), and the muscle layer (OVA vs no OVA)(p= 0.0001)(**Fig 1E**). Administration of the AMCase inhibitor allosamidin induced a significant decrease in esophageal eosinophils (**Fig 1)**. Allosamidin induced a 63 % decrease in eosinophil numbers in esophageal epithelium (OVA vs Allosamidin + OVA)(p=0.03)(**Fig 1C**), a 50 % decrease in eosinophils in the lamina propria (OVA vs Allosamidin + OVA)(p=0.04)(**Fig 1D),** and a statistically insignificant trend of fewer in eosinophils in the muscle layer (OVA vs Allosamidin + OVA)(p=0.8)(**Fig 1E).**

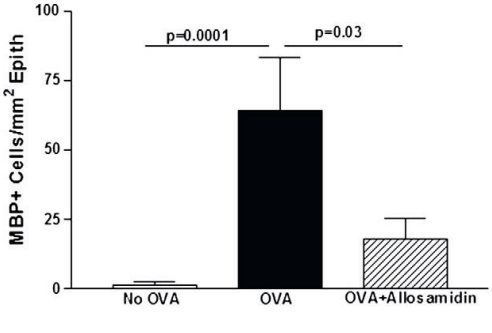


Figure 1 C

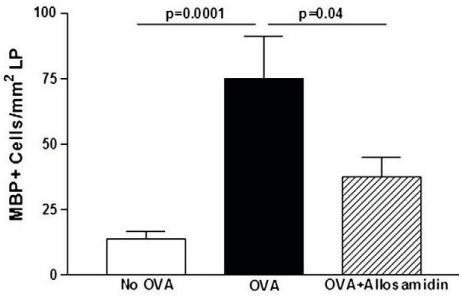


Figure 1D

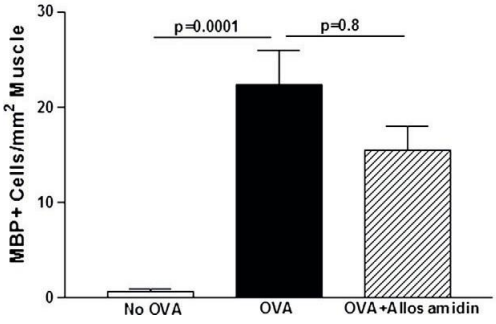
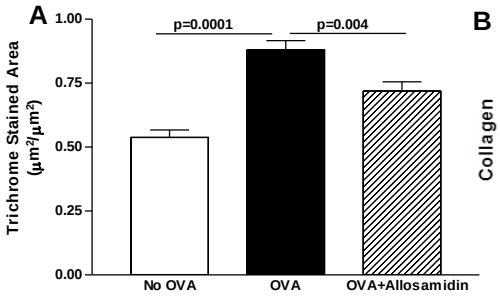
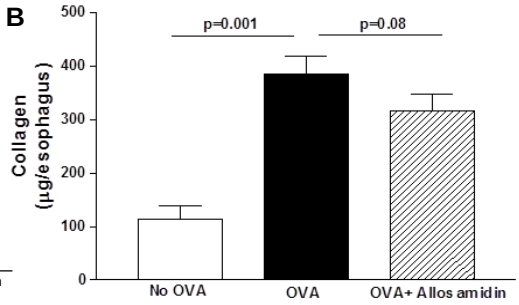


Figure 1E

Allosamidin inhibits oral OVA induced esophageal remodeling

Esophageal fibrosis: Oral OVA challenge induced a significant increase in esophageal fibrosis as assessed by either the area of esophageal trichrome staining (OVA vs no OVA)(p=0.0001)(**Fig 2A**) or the amount of esophageal collagen (OVA vs no OVA)(p=0.001)(**Fig 2B**). Allosamidin decreased the area of esophageal trichrome staining by 18% (OVA vs Allosamidin + OVA)(p=0.004)(**Fig 2A**). Although allosamidin also decreased the amount of esophageal collagen by a similar amount to that detected with trichrome staining (i.e. 18%), this decrease in collagen approached but did not reach statistical significance (OVA vs Allosamidin + OVA)(p=0.08)(**Fig 2B**).





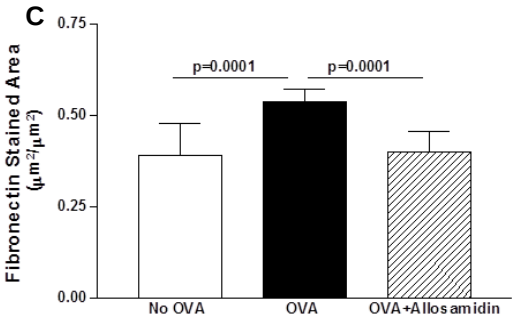


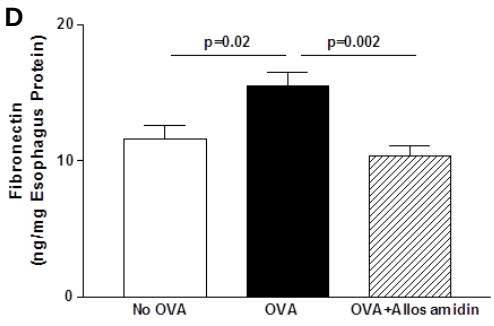
Figure 2A **Figure 2B** **Figure 2C**

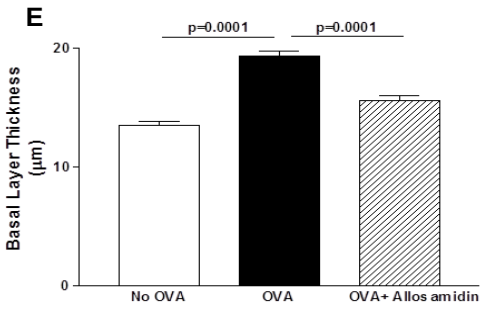
Esophageal deposition of fibronectin: Levels of deposition of the extracellular matrix protein fibronectin were significantly increased in the esophagus following oral OVA challenge as assessed by either the area of eosophageal fibronectin staining (OVA vs no OVA)(p=0.0001)(**Fig 2C**) or the amount of esophageal fibronectin as quantitated by ELISA (OVA vs no OVA)(p=0.02)(**Fig 2D**). Allosamidin decreased the area of esophageal fibronectin staining in oral OVA challenged mice (OVA vs Allosamidin + OVA)(p=0.0001)(**Fig 2C**), and the amount of esophageal fibronectin quantitated by ELISA (OVA vs Allosamidin + OVA)(p=0.002)(**Fig 2D**).

Esophageal epithelial basal zone hyperplasia: The thickness of the esophageal epithelial basal zone was significantly increased following OVA challenge (OVA vs no OVA)(p=0.0001)(**Fig 2E**). Allosamidin decreased the thickness of the esophageal epithelial basal zone (OVA vs Allosamidin + OVA)(p<0.0001)(**Fig 2E**),

Esophageal TGF-β1+ cells

The number of esophageal TGF-β1+ cells increased significantly in OVA challenged mice (p=0.01)(**Fig 2F**). Allosamidin decreased the number of esophageal TGF-β1+ cells (OVA vs Allosamidin + OVA)(p=0.05)(**Fig 2F**).





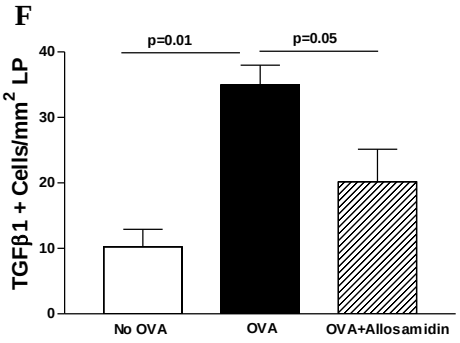


Figure 2D **Figure 2E** **Figure 2F**

Allosamidin inhibits oral OVA induced expression of eotaxin-1 in the esophagus

Oral OVA challenge induced a significant increase in esophageal eotaxin-1 (OVA vs no OVA)(p=0.01)(**Fig 3A)**, IL-13 (OVA vs no OVA)(p=0.004)(**Fig 3B**), but not IL-5 (OVA vs no OVA)(p=ns)(**Fig 3C**) as assessed by ELISA. Administration of allosamidin to oral OVA challenged mice significantly reduced levels of esophageal eotaxin-1 (OVA vs Allosamidin + OVA)(p=0.05)(**Fig 3A**). Allosamidin induced a trend for reduction in IL-13 which was not significant (OVA vs Allosamidin + OVA)(p=0.11)(**Fig 3B**), and no reduction in IL-5 (OVA vs Allosamidin + OVA)(p=ns).

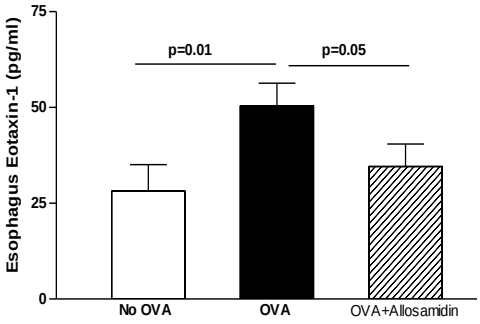


Figure 3A

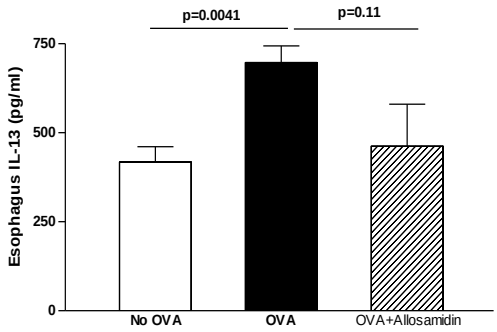


Figure 3B

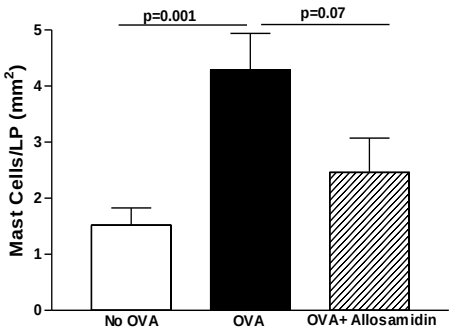


Figure 4

Effect of allosamidin on oral OVA induced mast cell inflammation in the esophagus

Oral OVA challenge induced a significant increase in mast cells in the lamina propria (p= 0.001)(**Fig 4**), but not the epithelium (data not shown). Administration of allosamidin to oral OVA challenged mice resulted in a trend for reduced numbers of mast cells that approached but did not reach statistical significance (OVA vs Allosamidin + OVA)(p=0.07)(**Fig 4**).

Effect of allosamidin on AMCase activity in the esophagus

The esophagus from non-OVA challenged mice expressed constitutive AMCase enzymatic activity (**Fig 5**). Following oral OVA challenge there was a slight but statistically significant increase in AMCase enzymatic activity in the esophagus (OVA vs no OVA)(p=0.03) (**Fig 5**). Administration of allosamedin significantly reduced AMCase activity in the esophagus (p=0.001)(**Fig 5**).

Effect of oral allosamidin on OVA induced esophageal eosinophilic inflammation

Oral administration of allosamidin significantly reduced the number of eosinophils in the esophageal epithelium of OVA challenged mice (OVA vs oral allosamidin + OVA)(p=0.03)(**Fig 6A**). While there was a trend for oral allosamidin to decrease the number of eosinophils in the lamina propria and smooth muscle this was not statistically significant. Oral administration of allosamedin did not inhibit basal layer thickness, or esophageal fibrosis as assessed by the area of trichrome staining.

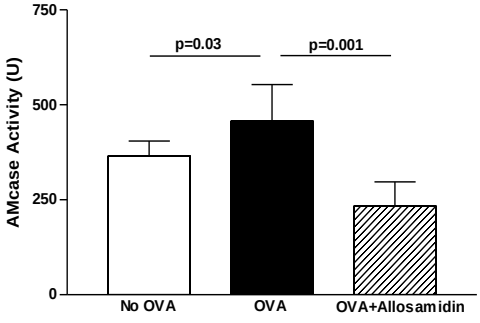


Figure 5

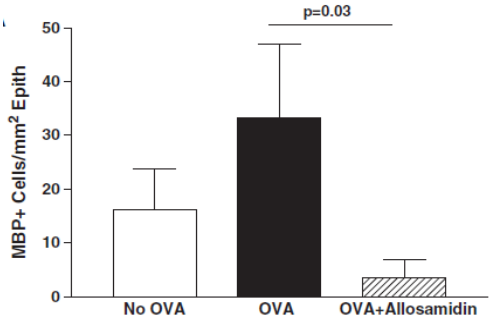


Figure 6A

Targeting AMCase study Key accomplishment conclusion:

Pharmacologic inhibition of AMCase with allosamidin inhibited both OVA induced increases in esophageal eosinophilic inflammation and OVA induced esophageal remodeling (fibrosis, epithelial basal zone hyperplasia, extracellular matrix deposition of fibronectin). This inhibition of eosinophilic inflammation in the esophagus by allosamidin was associated with reduced eotaxin-1 expression in the esophagus. These studies suggest that pharmacologic inhibition of AMCase results in inhibition of eosinophilic inflammation and remodeling in the esophagus in a mouse model of egg induced EoE partially through effects in the esophagus on reducing chemokines (i.e. eotaxin-1) implicated in the pathogenesis of EoE.

Reportable Outcomes:

- Smad-3 deficient mice have significantly less esophageal fibrosis in a mouse model of egg induced EE.
- Targeting chitinase with allosamidin reduced eosinophilic inflammation in a mouse model of egg induced EE.
- Targeting chitinase with allosamidin reduced esophageal fibrosis in a mouse model of egg induced EE.

Conclusion:

Targeting Smad3 or chitinase both partially reduced esophageal fibrosis. The mechanism by which targeting chitinase or Smad 3 reduces remodeling occurs at different steps in the eosinophil/TGF-b/Smad3 pathway as allosamidin inhibits both eosinophilic inflammation and fibrosis, whereas targeting smad3 only reduces fibrosis and does not effect numbers of eosinophils or TGFb+ cells. The combination of targeting Smad3 and chitinase may be more effective than targeting each pathway alone.

References:

None.

Appendices:

1. Abstract: Doshi et al (2013) listed below and attached. This abstract was presented at the AAAAI annual meeting in San Antonio in 2013.
2. Published Original Manuscript: Jae Youn Cho, Peter Rosenthal, Marina Miller, Alexa Pham, Seema Aceves, Shohei Sakuda, and **David Broide.** Targeting AMCase reduces esophageal eosinophilic inflammation and remodeling in a mouse model of egg induced eosinophilic esophagitis. *Int Immunopharmacology* (2014) 18: 35-42.
3. Revised Original Manuscript: Jae Youn Cho, Ashmi Doshi, Peter Rosenthal, Andrew Beppu, Marina Miller, Seema Aceves, **David Broide.** Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis. *Journal of Pediatric Gastroenterology & Nutrition*. (2014). Revised manuscript submitted

Manuscripts/Reprints, Abstracts:

Abstract

Ashmi Doshi, JaeYoun Cho, Peter Rosenthal, Seema Aceves, **David Broide.** Smad3 deficient mice have reduced esophageal fibrosis in a mouse model of eosinophilic esophagitis. *J Allergy Clinical Immunology* (2013), 507.

Original Manuscripts

Jae Youn Cho, Peter Rosenthal, Marina Miller, Alexa Pham, Seema Aceves, Shohei Sakuda, and **David Broide.** Targeting AMCase reduces esophageal eosinophilic inflammation and remodeling in a mouse model of egg induced eosinophilic esophagitis. *Int Immunopharmacology* (2014) 18: 35-42.

Jae Youn Cho, Ashmi Doshi, Peter Rosenthal, Andrew Beppu, Marina Miller, Seema Aceves, **David Broide.** Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis. *Journal of Pediatric Gastroenterology & Nutrition*. (2014). Revised manuscript submitted.

SUNDAY

505 Effect of Dermatophagoides Pteronyssinus Allergens On Expression of Genes Involved in Inflammation and Tissue Remodeling by Peripheral Blood Mononuclear Cells of Allergic Asthma Patients

Krzysztof Kowal, MD, PhD[1], Pawel Bernatowicz, MD[2], Lech Chyczewski, M.D., Ph.D.[2], Anna Bodzenta Lukaszyk, PhD[2]; [1]Medical University of Bialystok, Bialystok, Poland, [2]Medical University of Bialystok.

RATIONALE: Peripheral blood mononuclear cells (PBMC) participate in inflammatory response and tissue remodeling. The effect of D pteronyssinus (Dp) extract on expression of genes involved in inflammation and tissue remodeling by PBMC of Dp allergic asthma patients (APs) was evaluated.

METHODS: PBMC isolated from 30 Dp-APs and 10 healthy controls (HCs) were cultured with Dp, lipopolysaccharide (LPS) or without any stimulation. After 6 hours the cells were harvested for RNA isolation and after 24 hours the supernatants were collected for protein assessment. Expression of 84 genes was evaluated using SYBR Green-based microarray. The expression of the up-regulated genes was verified using TaqMan-based real-time PCR and by evaluation of the protein concentration using ELISA.

RESULTS: Stimulation with Dp induced significant (>2-fold) up-regulation of *CCL2, CCL3, IL-1beta, MMP-9* and *TSP-1* expression, both in HCs and Dp-AAs. The greatest up-regulation was demonstrated for *CCL2* (>12-fold). Stimulation with LPS caused significant up-regulation of *CCL2, CCL3, IFN-gamma, IL-1beta, IL-10 MMP-3, MMP-14, STAT-1* and *TSP-1* expression. The greatest up-regulation (>10-fold) was demonstrated for *CCL3, IL-1beta* and *IL-10*. Stimulated with Dp PBMC derived from Dp-AAs released more CCL2 than those derived from HCs. Stimulation with Dp resulted in greater release of CCL2 (7.05+/-4.8 ng/ml) than CCL3 (5.05+/-3.8 ng/ml; $p<0.05$), while LPS caused greater release of CCL3 (37.8+/-16.5ng/ml) than CCL2 (10.1+/-8.1 ng/ml; $p<0.05$).

CONCLUSIONS: Differential expression of genes involved in tissue remodeling and inflammation including *CCL2, CCL3* and *IL-10* by Dp and LPS stimulated PBMC may affect the course and outcome of the inflammatory response induced by those environmental factors.

506 Increased Prevalence of Compound Heterozygous Filaggrin Mutations in Severe Atopic Dermatitis in the United States

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RATIONALE: Filaggrin (FLG) mutations are the strongest genetic risk factor predisposing to atopic dermatitis (AD). Most studies have involved European cohorts analyzing for the 2 major FLG mutations. We aimed to investigate the frequency of FLG mutations in severe AD, and total serum IgE among genotypes in a cohort from regions throughout the United States.

METHODS: Patients referred to National Jewish Health for severe AD were genotyped for 5 FLG gene mutations (R501X, 2282del4, R2447X, S3247X, and 3702del4), and had total serum IgE level drawn.

RESULTS: 41/97 (42.2%) patients were found to have FLG mutations of which there were 5 (5.1%) homozygous (2 R501X/R501X and 3 2282del4/2282del4), 11 (11.3%) were compound heterozygous (seven R501X/2282del4, three 2282del4/R2447X, and one R501X/S3247X), and 25 (25.8%) heterozygous mutations (10 R501X, 11 2282del4, 3 R2447X, 1 S2347X). 4/11 (36.3%) compound heterozygous and 4/25 (16%) heterozygous mutations were identified by screening additional mutations. Serum IgE was greater in FLG null mutation patients when compared to wild type ($p=0.03$). Mean serum IgE for homozygous + compound heterozygous, heterozygous, and wild type genotype were 6037 kU/L, 3595 kU/L, and 1689 kU/L respectively.

CONCLUSIONS: This study demonstrates that FLG mutations are common in a U.S. cohort of severe AD patients. Interestingly there was a higher prevalence of compund heterozygous mutations than homozygous mutations. Those with homozygous or compound heterozygous mutations had higher IgE levels than the heterozygous or wild type genotype. The increased prevalence of FLG mutations in severe AD highlights the importance of this epidermal protein.

507 Smad-3 Deficient Mice Have Reduced Esophageal Fibrosis in a Model of Eosinophilic Esophagitis

Ashmi Doshi, MD[1], JaeYoun Cho, MD, PhD[1], Peter Rosenthal[1], Seema Sharma Aceves, MD, PhD, FAAAAI[2], David H. Broide, MB, ChB, FAAAAI[3]; [1]University of California, San Diego, [2]UCSD, Department of Pediatrics, La Jolla, CA, [3]Division of Allergy, Immunology.

RATIONALE: Eosinophilic Esophagitis (EoE) is an increasingly prevalent food triggered disease of adults and children and is associated with esophageal remodeling and stricture formation. In this study we utilized a murine model of egg (OVA) induced EoE in order to determine whether inhibiting TGF-b signaling through the Smad-3 pathway would inhibit esophageal remodeling.

METHODS: Wild type (WT) and Smad-3 deficient (KO) mice were sensitized to intraperitoneal OVA and challenged with intragastric OVA (starting on day 28) three times a week for 1 month. Control mice were sensitized and challenged with diluent. The esophagi from WT and Smad-3 KO mice were processed for immunohistochemistry and image analysis was utilized to quantitate features of esophageal remodeling, including the numbers of MBP+ eosinophils, TGF-b + cells, severity of fibrosis on trichrome stain, and severity of basal zone hyperplasia.

RESULTS: OVA challenge induced similar increases in MBP+ eosinophils and TGF-b positive cells in the esophagi of WT and Smad-3 KO mice. However, Smad-3 KO mice challenged with OVA had significantly less fibrosis compared to WT, as measured by the area of esophageal trichrome staining ($1.53\mu m^2/\mu m^2 \pm 0.11$ vs. $0.88\ \mu m^2/\mu m^2 \pm 0.09$; $p<0.001$) (WT vs. Smad-3 KO). There was a trend for OVA challenged Smad-3 KO to have reduced basal zone hyperplasia, but this was not statistically significant.

CONCLUSIONS: In a mouse model of egg induced EoE, Smad-3 deficient mice have significantly less esophageal remodeling, especially fibrosis. Targeting the TGF-b /Smad-3 pathway may be a novel way to reduce esophageal fibrosis and its associated complications such as esophageal strictures.

Journal of Pediatric Gastroenterology & Nutrition

Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis

--Manuscript Draft--

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Abstract:	Objectives: Eosinophilic esophagitis (EoE) is a food triggered disease associated with esophageal fibrosis and stricture formation in a subset of patients. In this study we utilized a murine model of egg (OVA) induced EoE to determine whether inhibiting TGF-β1 signaling through the Smad3 pathway would inhibit features of esophageal remodeling including fibrosis, angiogenesis, and basal zone hyperplasia. Methods: Wild type (WT) and Smad3 deficient (KO) mice were sensitized intraperitoneally and then challenged chronically with intra-esophageal OVA for one month. Levels of esophageal eosinophils, esophageal TGF-β1+ and VEGF+ cells, as well as features of esophageal remodeling (fibrosis, angiogenesis, basal zone hyperplasia) were quantitated by immunohistochemistry and image analysis. Results: OVA challenge induced a similar increase in the levels of esophageal MBP+ eosinophils and esophageal TGF-β1+ cells in WT and Smad3 KO mice. However, Smad3 KO mice challenged with OVA had significantly less esophageal fibrosis and esophageal angiogenesis compared to OVA challenged WT mice. The reduced esophageal angiogenesis in Smad3 KO mice was associated with reduced numbers of VEGF+ cells in the esophagus. There was a trend for OVA challenged Smad3 KO to have reduced basal zone hyperplasia, but this was not statistically significant. Conclusion: In a mouse model of egg induced EoE, Smad3 deficient mice have significantly less esophageal remodeling, especially fibrosis and angiogenesis which is associated with reduced expression of VEGF. Targeting the TGF-β1/Smad3 pathway

	may be a novel strategy to reduce esophageal fibrosis and its associated complications such as esophageal strictures in EoE.
Additional Information:	
Question	**Response**
What is the manuscript's word count?	2040
How many tables and figures are included?	6

Response to Reviewer #1:
Cho et al seek to expand their previous findings related to esophageal remodeling in their mouse model of EoE. Their model is the only mouse model based on food sensitization and challenge and their findings provide further support for the impact of the Smad pathway in the genesis of remodeling.

Criticisms
Comment 1. Page 8. the authors state they will look at angiogenic cytokines and only describe VEGF positive cells. Were there others examined?
Response: As VEGF is recognized as the key angiogenic cytokine in many diseases associated with angiogenesis, and as we have previously demonstrated increased levels of VEGF+ cells in this mouse model of OVA induced EoE (Rubenstein E et al JPGN, 53:409-416, 2011), we focused this study on the effect of Smad 3 on VEGF+ cells. These studies demonstrated that levels of VEGF+ cells and angiogenesis were significantly reduced in Smad3 KO mice. We recognize that this does not indicate that VEGF is the cause of angiogenesis in EoE, and indicate in the revised Discussion " that further studies will need to be performed using inhibitors of VEGF and/or other angiogenic cytokines to determine their individual roles in contributing to angiogenesis in EoE".

Comment 2. Page 10. Extensions of a pre-clinical model to esophageal strictures and food impactions are not supported here and should be tempered somewhat. This is a critical area of discover that the investigators are leaders in but the model as presented demonstrates molecular insights into the pathogenesis of remodeling but not necessarily its functional impact.
Response: We agree and have added this caution to the Discussion.

Comment 3. As the authors only report IHC data per unit area, it would be helpful to reiterate the method of selection of the area measured. Was this the most densely stained area? Would be helpful to address this as no other measure of these mediators is presented.
Response: For IHC experiments, the total area of lamina propria in each slide was counted and results expressed per mm^2 of lamina propria. This has been added to the Methods.

Comment 4. Figure 1. Levels of eosinophilia in the Smad2 KO in figure 1d do not appear consistent with that shown numerically in 1e. 1b appears to have many more cells thank 1d. can the authors presetn a more representative figure for 1d? Maybe 1 b has more degranulation than 1d.
Response: We have replaced Figure 1 with more representative figures.

Comment 5. Figure 3/4. Do Smad3 KO naive mice have less trichrome staining / blood vessels than WT naive? Could this explain the difference in trichrome / blood vessel staining?
Response: As described in the Methods, to enhance the ability to detect new vessels, only those small blood vessels $\leq$5 microns were defined as angiogenic and were the blood vessels counted. These very small blood vessels have minimal trichrome staining in WT mice. We did not note differences in WT vs Smad 3 KO mice in the very low levels of trichrome staining in small blood vessels $\leq$5 microns. We detected blood vessels using PECAM staining (which does not detect trichrome), so even if there were a small difference in trichrome staining of WT and SMAD3 KO blood vessels, this would not influence these results.

Comment 6. Figure 6. What does this add to the manuscript? There is a mismatch between the text and the figure. is there a difference between WT and Smad3 KO with respect to hyperplasia? If so, this adds to the role of Smad3 with respect to remodeling. If not, the findings could be stated in words.

Response: There is no significant difference in basal zone hyperplasia in WT and Smad3 KO mice. We have stated this in the text and deleted Fig 6 as requested.

Response to Reviewer #2:
General Comments.
General Comment 1.
In this study, the authors have investigated whether inhibiting TGF-b1 signaling via Smad3 pathway would reduce tissue remodeling, namely fibrosis, angiogenesis, and basal layer hyperplasia in a mouse EoE model. Compared to WT control, they have shown that Smad3 deficient mice have less esophageal remodeling after OVA sensitization and repeated challenges.
Response: Thank you.

General Comments 2.
In essence, the results are somewhat expected. The direct link between TGF-b1 and EoE-induced tissue remodeling requires more validation. It is known that Smad3 activation can be influenced by other signaling molecules such as Smad7 or Akt, and other cytokines or growth factors could indirectly affect Smad3 signaling. Therefore to ascertain the direct effect of TGF-b1 signaling, it would be important to compare the cells such as peripheral/bone marrow-derived eosinophils/mast cells between WT and Smad3 KO mice in terms of their response to recombinant TGF-b1 in a culture system.

Response: We have cultured purified populations of bone marrow derived mast cells in vitro derived from WT and Smad3 KO mice in the presence or absence of TGF-b as requested by the reviewer. Previous studies culturing mast cells in vitro with TGF-b from our laboratory have demonstrated that TGF-b inhibits mast cell proliferation, but does not influence pre-formed granule beta hexoseaminidase release, or LTC4 generation (Broide D, et al. Transforming growth factor-beta 1 selectively inhibits IL-3-dependent mast cell proliferation without affecting mast cell function or differentiation. J Immunol. 1989 ;143:1591-7). We therefore examined the effect of TGF-b1 on mast cell proliferation. TGF-b1 inhibited WT mast cell proliferation by 47 %, and Smad3 KO mast cell proliferation by 18% ($P < 0.05$). Thus, Smad 3 plays a significant role in mediating the effect of TGFb1 on mast cells. Our in vitro studies with Smad3 deficient mast cells confirm that Smad3 plays a significant role in mediating the effect of TGFb1, although a minor inhibitory effect of TGF-b1 that was Smad3 independent was also noted. We have added this to the Methods, Results, and Discussion.

General Comment 3.
In addition, the authors should compare the degree of esophageal inflammation and IL-5/eotaxin expression between WT and Smad3 KO. If no difference of inflammation is observed, this would suggest thatTGF-b1/Smad3 signaling is more specific for tissue remodeling. Lastly, the authors need to discuss statistical analysis in the Method.
Response:

a) There are no differences in esophageal eosinophilic inflammation or IL-5 between WT and Smad3 KO mice. Eotaxin-1 was not detected in either WT or Smad3 KO mice. This is included in the Results.
b) The statistical analysis is in the “Data Analysis” section on p13 according to the style of this journal

There are a number of technical points:

Comment 1. Fig. 1. The histology images are blurry, and high power field image might provide better resolution. Although OVA treatment increased eosinophils in the esophagus, a majority of eosinophilis appear to be located in the lumen and submucosal layer, and there is no significant intraepithelial infiltration, which is often seen in human EoE and murine models published by Rothenberg group. Furthermore, please comment on the location (proximal, mid, or distal esophagus) where the histology specimens were taken.

Response:

a) Figure 1.

We have provided new Figure 1 images.

b) Intraepithelial eos

We agree that the majority of eosinophils in the OVA model of EoE we have used are located in the submucosal layer. All mouse models of EoE have limitations compared to human EoE. The advantage of the OVA model we use is that it reproduces an oral food antigen administered model of EoE which administers food antigens the one stimulus known to be a key factor in the pathogenesis of EoE. While other mouse models (transgene, fungus) may produce greater intra-epithelial eosinophilic inflammation they are less "physiologic" when compared to administering an oral food to induce EoE.

c) Histology

The Methods (p 5) describe that the histology from each section (proximal, mid, and distal esophagus) is included in the analysis of each mouse. Results in each group are presented as a combined score of the 3 layers analyzed (upper, middle, lower).

Comment 2. Fig. 2. TGF-b1 staining estimates the number of TGF-producing cells, and does not accurately reflect the amount of TGF produced by these cells. Therefore, the authors should include RT-PCR and/or western blot data to compare esophageal TGF-b1 level in these mice.

Response: We have added new Fig 2B demonstrating by qPCR that OVA induces increased levels of TGF-b1 mRNA in the esophagus of both WT and Smad3 KO mice

Comment 3. Figs 3, 4, and 5. I would suggest including IHC images. In addition, have the investigators examined the expression of collagen subtypes or other angiogenic factors? In Fig. 5, the baseline level of VEGF+ cells in Smad3 KO seems to be higher than that in WT. Is this difference statistically significant?

Response:

a) We have added images for Figures 3-5
b) We have not investigated the expression of collagen subtypes or other angiogenic factors
c) In Fig 5 there is no statistical difference in the baseline

Comment 4. Fig. 6. The authors should include representative histology of H&E staining so that the readers can correlate basal layer hyperplasia with eosinophilia. Fig. 1 does not seem to show marked basal layer hyperplasia in OVA-treated WT group.

Response: We have removed Fig 6 and reported the data in the text as requested by Reviewer #1.

Smad3 deficient mice have reduced esophageal fibrosis and angiogenesis in a mouse model of egg induced eosinophilic esophagitis

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ABSTRACT

Objectives: Eosinophilic esophagitis (EoE) is a food triggered disease associated with esophageal fibrosis and stricture formation in a subset of patients. In this study we utilized a murine model of egg (OVA) induced EoE to determine whether inhibiting TGF-β1 signaling through the Smad3 pathway would inhibit features of esophageal remodeling including fibrosis, angiogenesis, and basal zone hyperplasia.

Methods: Wild type (WT) and Smad3 deficient (KO) mice were sensitized intraperitoneally and then challenged chronically with intra-esophageal OVA for one month. Levels of esophageal eosinophils, esophageal TGF-β1+ and VEGF+ cells, as well as features of esophageal remodeling (fibrosis, angiogenesis, basal zone hyperplasia) were quantitated by immunohistochemistry and image analysis.

Results: OVA challenge induced a similar increase in the levels of esophageal MBP+ eosinophils and esophageal TGF-β1+ cells in WT and Smad3 KO mice. However, Smad3 KO mice challenged with OVA had significantly less esophageal fibrosis and esophageal angiogenesis compared to OVA challenged WT mice. The reduced esophageal angiogenesis in Smad3 KO mice was associated with reduced numbers of VEGF+ cells in the esophagus. There was a trend for OVA challenged Smad3 KO to have reduced basal zone hyperplasia, but this was not statistically significant.

Conclusion: In a mouse model of egg induced EoE, Smad3 deficient mice have significantly less esophageal remodeling, especially fibrosis and angiogenesis which is associated with reduced expression of VEGF. Targeting the TGF-β1/Smad3 pathway may be a novel strategy to reduce esophageal fibrosis and its associated complications such as esophageal strictures in EoE.

Key words: eosinophil, fibrosis, fibronectin,

INTRODUCTION

Eosinophilic esophagitis (EoE) is characterized histologicaly by a dense esophageal eosinophilia (>15 eos/HPF), and clinically by the presence of symptoms related to esophageal dysfunction including dysphagia, chest pain, abdominal pain, and food impactions (1, 2). Food impactions are related to esophageal strictures which develop in a subset of subjects with EoE (3,4, 5). In this study we have investigated the role of TGF-β1 and the Smad 3 pathway which mediates TGF-β1 intracellular signal transduction (6) in contributing to esophageal fibrosis and remodeling using a mouse model of oral food induced EoE which is associated with esophageal fibrosis and remodeling including angiogenesis, and deposition of extracellular matrix components such as fibronectin (7). We have focused on the TGF-β1/Smad3 pathway in this study as TGF-β1 is a cytokine that is highly expressed in the esophagus in EoE (8,9,10,11,12), and the TGF-β1/Smad3 pathway is known to be important in mediating fibrosis in many diseases associated with fibrosis (6).

In EoE, eosinophils (8) and mast cells (9) are a prominent source of TGF-β1 in the esophagus. To contribute to esophageal fibrosis in EoE, TGF-β1 released from eosinophils, mast cells, or other TGF-β1 producing cells would need to bind to TGF-β1 cell surface receptors (TGF-βRI and TGF-βRII) on fibroblasts which would subsequently activate intracellular Smads to translocate to the nucleus to active transcription of collagen genes which contribute to fibrosis (6). Smad proteins are thus a family of transcription factors that mediate TGF-β1 signals. The binding of active TGF-β1 to the TGF-β1 cell surface receptor results in phosphorylation of receptor regulated Smad2/3 (6). Once phosphorylated, Smad2/3 forms a complex with Smad4 which then translocates from the cytoplasm to the nucleus where it regulates transcription of collagen genes. Studies of human fibroblasts have identified a number of collagen gene promoters that

are induced by TGF-β1 and dependent on Smad 3 (13). Activation of these collagen gene promoters by TGF-β1 can be blocked by dominant-negative Smad3 expression vectors (13). Therefore, inhibition of TGF-β1/Smad3 signaling may directly reduce the expression of collagen-producing genes and resultant fibrosis. The importance of this TGF-β1 /Smad3 pathway to inhaled allergen induced airway fibrosis and remodeling of the airways in asthma has been demonstrated in mouse models of asthma using either Smad3 deficient mice (14) or anti-TGF-β1 Abs (15). In this study we demonstrate that using a mouse model of egg induced EoE, Smad3 deficient mice have significantly less esophageal remodeling, especially fibrosis and angiogenesis. Targeting the TGF-β1/Smad3 pathway in EoE may thus be a novel way to reduce esophageal fibrosis and its associated complications such as esophageal strictures.

MATERIALS AND METHODS

Oral OVA allergen induced esophageal eosinophilic inflammation

Eight week old female Smad3 deficient on a BALB/c background (kindly provided by Dr. Stuelten, NCI, with the permission of Dr Lise Wogensen, Arhus, Denmark who generated the mice) and control BALB/c mice (12 mice/group; The Charles River Laboratory, Wilmington, MA) were sensitized intraperitoneally on day 0 and 14 (50 µg of OVA adsorbed to 1 mg of aluminum hydroxide adjuvant in phosphate buffered saline (PBS), Sigma-Aldrich, St. Louis, MO) and challenged intra-esophageally three times/week for four weeks with 10 mg OVA suspended in 100 µl PBS on days 28, 30, 32, 35, 37, 39, 42, 44, 46, 49, 51, 53 as previously described (7). OVA was administered through an intragastric feeding needle (20-gauge, 1.5-inch; Pepper and Sons, Inc, New Hyde Park, NY). Mice were sacrificed 24 hrs after last administration of intraesophageal OVA (day 54). Control BALB/c mice were neither sensitized nor challenged. The esophagus was removed in its entirety and fixed with 4% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA) for 24 hrs., oriented and embedded in 1% agarose (Invitrogen, Carlsbad, CA), and then sectioned (upper, middle and lower). Five-micron esophageal sections were then prepared from each layer and equivalent numbers of sections from each layer were included in every experiment for analysis. Results in each group are presented as a combined score of the 3 layers analyzed (upper, middle, lower) as previously described (7). For immunohistochemistry experiments, the total area of lamina propria in each slide was counted and results expressed per mm^2 of lamina propria.

MBP+ esophageal eosinophils

Eosinophils were detected in esophageal tissue by immunohistochemistry using an anti-mouse Major Basic Protein (MBP) antibody (kindly provided by James Lee PhD, Mayo

Clinic, Scottsdale, AZ) as previously described (7). Quantitation of the number of eosinophils was performed using a light microscope attached to an image-analysis system with the entire cross section of the esophagus visualized. The area of the esophageal lamina propria analysis was outlined and this area determined by the image analysis software (Image-Pro Plus; Media Cybernetics). Results are expressed as the number of eosinophils per mm² of lamina propria.

Esophageal IL-5, eotaxin-1, and TGF-β1+ cells

Esophageal tissue sections were processed for immunohistochemistry using a primary mAb directed against either TGF-β1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), IL-5 (R&D), or eotaxin-1 (R&D), as described (16). Results are expressed as the number of IL-5, eotaxin-1, or TGF-β1 positive cells per mm² of lamina propria.

Esophageal TGF-β1 mRNA

The esophagus derived from WT and Smad3 deficient mice were processed for qPCR to detect TGF-β1 mRNA as previously described in this laboratory (17). In brief, total RNA was extracted with RNA-STAT-60 (Tel-Test) and reverse transcribed with Oligo-dT and SuperScript II kit (Life Technologies). qPCR was performed with TaqMan PCR Master Mix and TGF-β1, primers (Applied Biosystems). The relative amounts of transcripts were normalized to those of housekeeping gene (GAPDH) mRNA and compared by the $\Delta\Delta$Ct method as previously described in this laboratory (17).

Esophageal fibrosis

The area of trichrome staining in paraffin embedded esophagus was outlined and quantified using a light microscope attached to an image analysis system as previously

described (7). Results are expressed as the area of trichrome staining per μm length of basement membrane.

Esophageal angiogenesis and VEGF + cells

Blood vessels in esophageal tissue were identified by immunohistochemistry using a rat anti-mouse PECAM monoclonal antibody (BD Bioscience, San Jose, CA) which detects the blood vessel adhesion molecule PECAM as previously described in this laboratory (7). To enhance the ability to detect new vessels, only those small blood vessels ≤5 microns were counted as previously described in this laboratory (7). Results are expressed as the number of PECAM-1 positive vessels per mm² of lamina propria.

In addition we quantitated the number of VEGF positive cells using an anti-VEGF primary Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Results are expressed as the number of VEGF positive cells per mm² of lamina propria.

Esophageal Basal Zone Thickness

The epithelial basal zone thickness was assessed in esophageal sections stained with hematoxylin and eosin using a light microscope attached to an image-analysis system (7). The maximal thickness of the basal layer in each slide was recorded in μm.

Effect of TGF-β1 on Smad3 deficient versus WT mast cell proliferation

To determine whether Smad3 deficient cells have a reduced capacity to respond to TGF-β1, purified populations of mouse bone marrow derived mast cells (MBMMC) (> 99% pure) from WT and Smad3 deficient mice were cultured in triplicate in the presence or absence of TGF-β1 (10 ng/ml) for 48 hrs as previously described in this laboratory (18). The number of MBMMC in WT and Smad3 deficient cultures were quantitated

using the CyQUANT cell proliferation assay kit (Invitrogen). We have previously demonstrated that TGF-β inhibits WT MBMMC proliferation (18). Results are expressed as the % inhibition of MBMMC proliferation quantitated as (Baseline number of MBMMC-48 hour number of MBMMC) divided by 100.

RESULTS

MBP+ esophageal eosinophils

The number of esophageal eosinophils increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (122.9 ± 24.8 vs. 12.2 ± 2.2 eosinophils/mm^2; p<0.0001)(**Figure 1**). OVA challenge in Smad3 deficient mice induced a similar increase in the number of esophageal eosinophils (OVA Smad3 KO vs no OVA Smad3 KO; p<0.0001) as noted in OVA challenged WT mice (**Figure 1**).

Esophageal IL-5+ cells and eotaxin+ cells

There was no difference in the number of IL-5 positive cells in OVA challenged WT mice compared to OVA challenged Smad3 KO mice (0.72 ± 0.33 vs 0.85 ± 0.45 IL-5 positive cells/mm^2; p=ns). Eotaxin-1 positive cells were not detected in either WT or Smad3 KO mice.

Esophageal TGF-β1+ cells and esophageal TGF-β1 mRNA

The number of esophageal TGF-β1+ cells increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (p<0.01)(**Figure 2A**). There was no significant difference in the number of esophageal TGF-β1+ cells in OVA challenged Smad3 deficient mice compared to OVA challenged WT mice (p=ns)(**Figure 2A**).

Levels of esophageal TGF-β1 mRNA increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (p<0.01)(**Figure 2B**). There was no significant difference in levels of esophageal TGF-β1 mRNA in OVA challenged Smad3 deficient mice compared to OVA challenged WT mice (p=ns)(**Figure 2B**).

Esophageal Fibrosis

The area of esophageal trichrome staining (which detects collagen) increased significantly in WT mice challenged with OVA compared to non-OVA challenged mice (1.52 $\pm$ 0.10 vs. 0.54 $\pm$ 0.05 $\mu m^2/\mu m^2$ area of trichrome staining)($p<0.0001$)(**Figure 3**). Smad3 deficient mice challenged with OVA had a significant reduction in the area of esophageal trichrome staining compared to OVA challenged WT mice ($p<0.0001$)(**Figure 3**).

Esophageal angiogenesis

WT mice challenged with OVA had a significant increase in the number of esophageal small blood vessels as quantitated by PECAM staining ($p<0.0001$ vs no OVA)(**Figure 4**). In contrast, OVA challenged Smad3 deficient mice had a significant reduction in the number of esophageal small blood vessels compared to OVA challenged WT mice ($p< 0.0001$)(**Figure 4**).

Esophageal VEGF positive cells

To determine whether the Smad3 pathway influenced levels of angiogenic cytokines, we quantitated the number of VEGF positive cells within the lamina propria. WT mice challenged with OVA had a significant increase in the number of VEGF positive cells in the LP ($p<0.0001$; vs WT no OVA)(**Figure 5).** Smad3 deficient mice challenged with OVA had significantly reduced numbers of VEGF positive cells in the LP ($p<0.0001$; vs WT OVA) (**Figure 5**).

Esophageal Basal Zone Thickness

OVA challenged WT mice had a slight increase in basal zone thickness compared to non-OVA challenged WT mice which was not statistically significant (p=0.12). OVA challenged Smad3 deficient mice had a trend for reduction in basal layer thickness compared to OVA challenged WT mice which was not statistically significant (p=0.40).

Effect of TGF- β1 on Smad3 deficient versus WT mast cell proliferation

WT MBMMC cultured in the presence of TGF-β1 for 48 hours had a significantly greater inhibition of MBMMC proliferation (48% inhibition) compared to Smad3 deficient MBMMC cultured in the presence of TGF-β1 (18 % inhibition) ($p < 0.001$)(**Figure 6).**

DISCUSSION

In this study we have used a mouse model of egg induced EoE and esophageal remodeling to demonstrate the importance of the Smad3 signaling pathway to esophageal fibrosis and angiogenesis which are features of esophageal remodeling in EoE. As esophageal strictures and food impactions are an important complication of EoE (3,4,5), understanding the mechanism by which esophageal fibrosis and strictures are induced in pre-clinical mouse models of EoE provides insight into potential novel targets such as the TGF-β1/Smad3 signaling pathway in the subset of EoE subjects with esophageal strictures. The potential relevance to human EoE of our findings related to the Smad3 pathway in the mouse model of EoE is supported by studies of esophageal biopsies in EoE demonstrating increased numbers of esophageal TGF-β1+ cells (including eosinophils and mast cells expressing TGF-β1)(8,9,10,11,12), activation of the Smad3 signaling pathway (8), increased fibrosis (8,9,10), and increased angiogenesis (8). While the pre-clinical model of EoE we have used in this study provides important insight into many features of OVA induced esophageal remodeling associated with EoE, we are not able to model esophageal strictures and food impactions with this mouse model.

Our studies in Smad3 deficient mice demonstrated that they have similar levels of esophageal eosinophilic inflammation and esophageal TGF-β1+ cells as WT mice when challenged with OVA. Thus, targeting the Smad3 pathway does not inhibit the ability of eosinophils to traffick from the bone marrow to the esophagus, or inhibit the ability of cells in the esophagus such as eosinophils and mast cells to express TGF-β1. However, targeting the Smad3 pathway does inhibit the ability of cells which express TGF-β receptors coupled to intracellular Smad3 signal transduction pathways to respond to TGF-β1 released by eosinophils and mast cells. Thus, in Smad3 deficient mice esophageal fibroblasts (which highly express TGF-β receptors that are coupled to

intracellular Smad3 signal transduction pathways), are unable to respond to TGF-β1 to express collagen which results in less esophageal fibrosis. Our in vitro studies with Smad3 deficient mast cells confirm that Smad3 plays a significant role in mediating the function of TGF-β1, although a minor inhibitory effect of TGF-β1 that was Smad3 independent was also noted.

Our studies also demonstrated that Smad3 deficient mice have reduced esophageal angiogenesis another feature of esophageal remodeling noted in esophageal biopsies from subjects with EoE (8). Angiogenic vessels are known to exhibit increased expression of adhesion molecules (19) and may contribute to increased inflammation through increased recruitment of eosinophils into the esophagus. Studies in EoE have demonstrated increased angiogenic blood vessels with increased levels of expression of the adhesion molecule VCAM-1 (8) which binds VLA-4 expressed by eosinophils. As VEGF is a key mediator of angiogenesis (20), we quantitated levels of VEGF positive cells in the esophagus in WT and Smad3 deficient mice. These studies demonstrated that OVA challenge induced increased numbers of VEGF + cells and angiogenesis in the esophagus in WT mice, while levels of VEGF+ cells and angiogenesis were significantly reduced in Smad3 KO mice. The mechanism by which Smad3 influences levels of VEGF is likely to be indirect as the Smad3 transcription factor is not a key regulator of VEGF transcription. However, there is considerable evidence that TGF-β1 (which we have detected at increased levels in EoE and which signals through Smad3) can mediate angiogenesis in vivo (21,22,23,24). Because of TGF-β1's inhibitory effects on endothelial cells in vitro (25), TGF-β1 is hypothesized to induce angiogenesis *in vivo* through an indirect mechanism, by inducing expression of VEGF and/or other angiogenic factors (25). For example TGF-β1 deficient mice die *in utero* and show defective vasculogenesis (21). Similarly mice deficient in the TGF-β1 receptor I (ALK1

deficient mice) die from defects in angiogenesis (21,22,23). In addition, *in vivo* administration of TGF-β1 subcutaneously in newborn mice induces angiogenesis (24). As levels of VEGF were reduced in the OVA challenged Smad3 deficient (who had reduced angiogenesis), it suggests that TGF-β1 is indirectly inducing angiogenesis in this model most likely mediated by angiogenic cytokines such as VEGF. However, further studies will need to be performed using inhibitors of VEGF and/or other angiogenic cytokines to determine their individual roles in contributing to angiogenesis in EoE.

In summary, we have demonstrated that in a mouse model of OVA food induced EoE that Smad3 deficient mice have significantly less esophageal remodeling, especially fibrosis and angiogenesis. In addition, reduced angiogenesis in Smad3 deficient mice was associated with reduced levels of expression of VEGF. Targeting the TGF-β1/Smad3 pathway may thus be a novel strategy to reduce esophageal fibrosis and its associated complications such as esophageal strictures in EoE.

DATA ANALYSIS

Results were compared by a Mann-Whitney test using a statistical software package (GraphPad Prism, San Diego, CA). P values <0.05 were considered statistically significant. Results are presented as the mean ± SEM.

ABBREVIATIONS

EoE: Eosinophilic esophagitis

MBMMC: Mouse bone marrow derived mast cell

MBP: Major Basic Protein

OVA: Ovalbumin

PECAM: Platelet endothelial cell adhesion molecule

VEGF: Vascular endothelial growth factor

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FIGURE LEGENDS

Figure 1: **Eosinophils in the Esophagus**

Eosinophils in the esophagus were detected using immunohistochemistry staining with an anti-Major Basic Protein antibody. A) WT No OVA. B) WT OVA. C) Smad3 KO no OVA. D) Smad3 KO OVA. E) OVA challenge induced a significant increase in esophageal eosinophils in WT mice (OVA vs no OVA; $p<0.0001$), as well as in Smad3 KO mice (OVA vs no OVA; $p<0.0001$).

Figure 2: **Esophageal TGFβ1+ cells and Esophageal TGFβ1 mRNA**

(A). TGFβ1 positive cells in the esophagus were detected using immunohistochemistry staining with an anti-TGFβ1 antibody. OVA challenge induced a significant increase in esophageal TGFβ1 positive cells in WT mice (OVA vs no OVA; $p=0.01$). There was no difference in numbers of TGFβ1 positive cells in OVA challenged Smad3 KO mice compared to OVA challenged WT mice (p=NS).

(B). The esophagus derived from WT and Smad3 deficient mice were processed for qPCR to detect TGF-β1 mRNA. The relative amounts of transcripts were normalized to those of housekeeping gene (GAPDH) mRNA.

Figure 3: **Esophageal fibrosis**

The area of esophageal trichrome staining was quantitated using light microscopy and image analysis. OVA challenge induced a significant increase in the area of esophageal trichrome staining in WT mice (OVA vs no OVA; $p<0.0001$). There was a significant reduction in the area of esophageal trichrome staining in OVA challenged Smad3 KO mice compared to OVA challenged WT mice ($p<0.0001$).

Figure 4: **Esophageal angiogenesis**

Blood vessels <5 um in diameter in the esophagus were detected using immunohistochemistry staining for PECAM. OVA challenge induced a significant increase in esophageal small blood vessels in WT mice (OVA vs no OVA; $p<0.0001$). There was a significant reduction in the number of esophageal small blood vessels in OVA challenged Smad3 KO mice compared to OVA challenged WT mice ($p<0.0001$).

Figure 5: **Esophageal VEGF expression**

VEGF positive cells in the esophagus were detected using immunohistochemistry staining with an anti- VEGF antibody. OVA challenge induced a significant increase in the number of esophageal VEGF+ cells in WT mice (OVA vs no OVA; $p< 0.0001$). There was a significant reduction in the number of esophageal VEGF+ cells in OVA challenged Smad3 KO mice (OVA WT vs OVA Smad3 KO; $p<0.0001$).

Figure 6: **Effect of TGF-β1 on Smad3 deficient versus WT mast cell proliferation**

Mouse bone marrow derived mast cells (MBMMC) (> 99% pure) from WT and Smad3 deficient mice were cultured in triplicate in the presence or absence of TGF-β1 (10 ng/ml) for 48 hrs. The number of MBMMC in WT and Smad3 cultures were quantitated using a CyQUANT cell proliferation assay kit. Results are expressed as the % inhibition of MBMMC proliferation.

STATEMENT OF CONTRIBUTION

Ja Youn Cho, Ashmi Doshi, Peter Rosenthal, Andrew Beppu, and Marina Miller, designed experiments, and performed experiments. Seema Aceves, and David Broide contributed to the design of the study, the interpretation of results, and the writing of the manuscript. All authors reviewed the final draft of the manuscript.

STATEMENT OF CONFLICTS OF INTEREST

None of the authors have a conflict of interest.

Figure 1

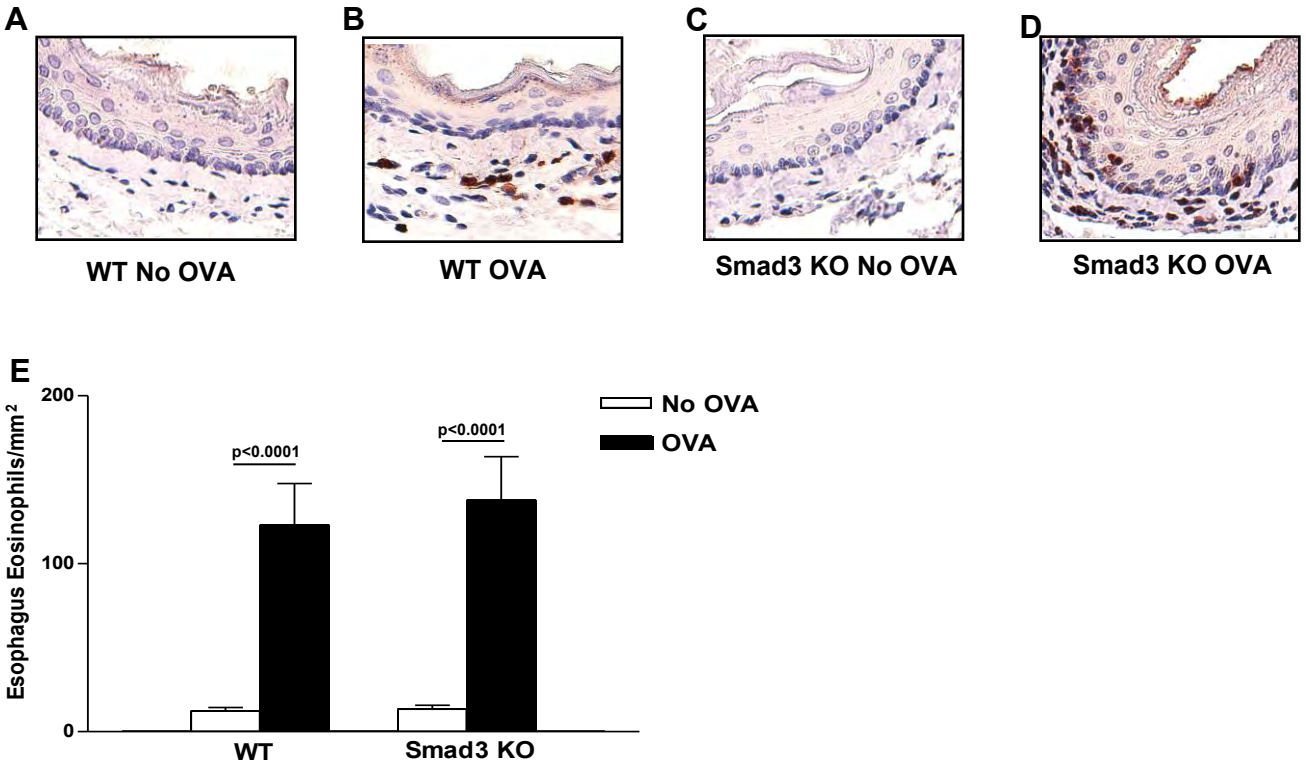


Figure 1

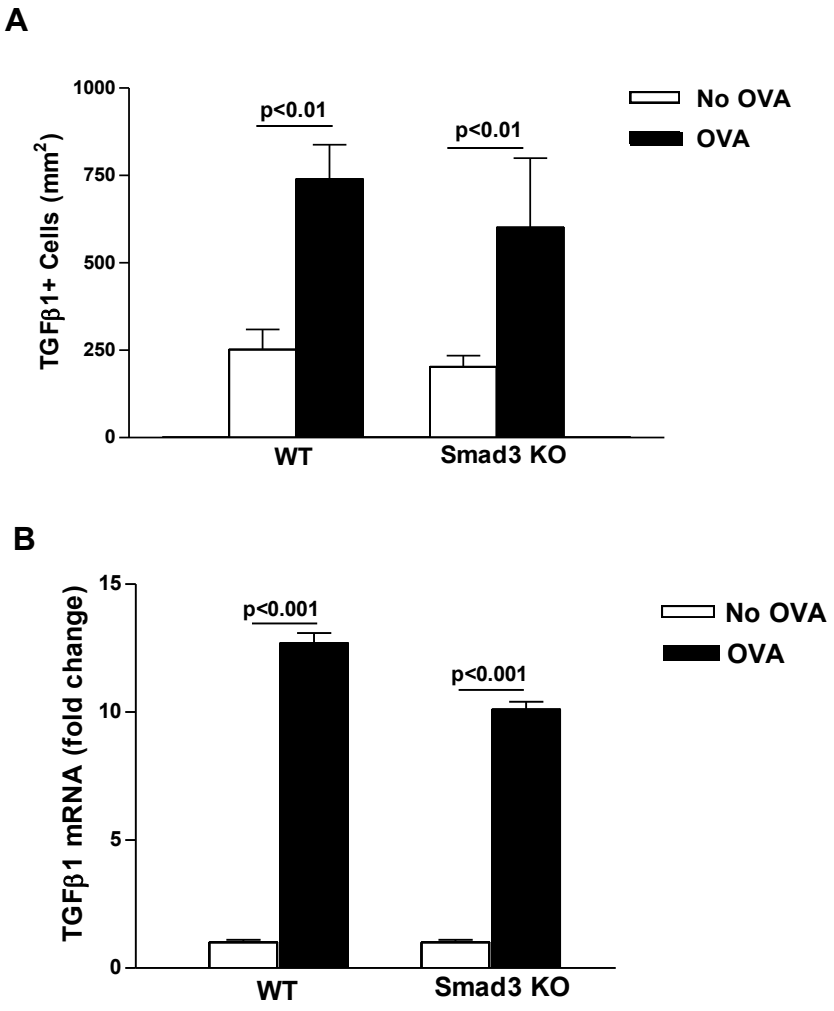
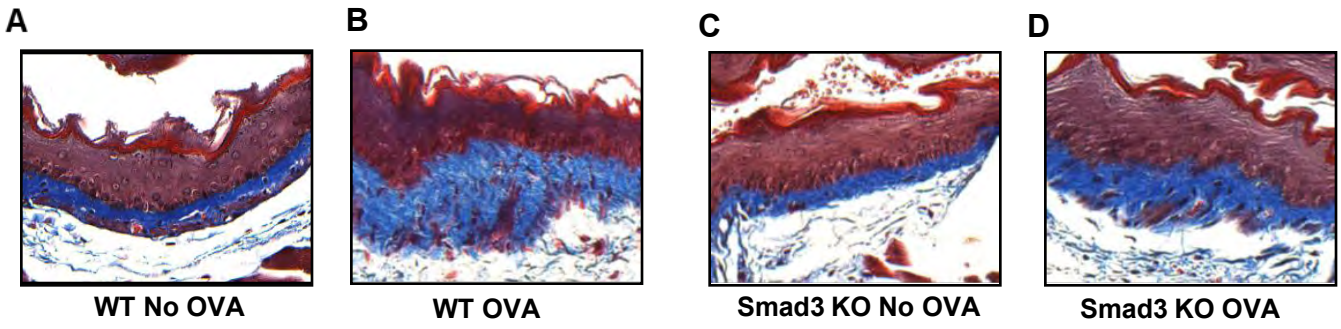

A
1000
750
500
250
0
TGFβ1+ Cells (mm2)
p<0.01
p<0.01
No OVA
OVA
WT
Smad3 KO
B
15
10
5
0
TGFβ1 mRNA (fold change)
p<0.001
p<0.001
No OVA
OVA
WT
Smad3 KO

Figure 2

Figure 3



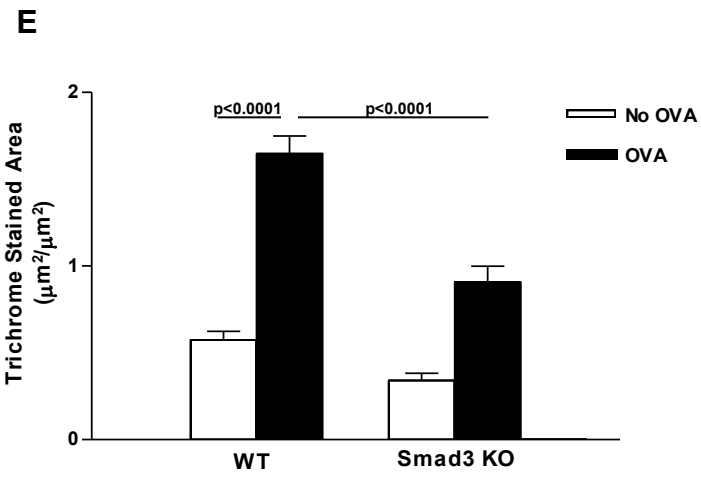
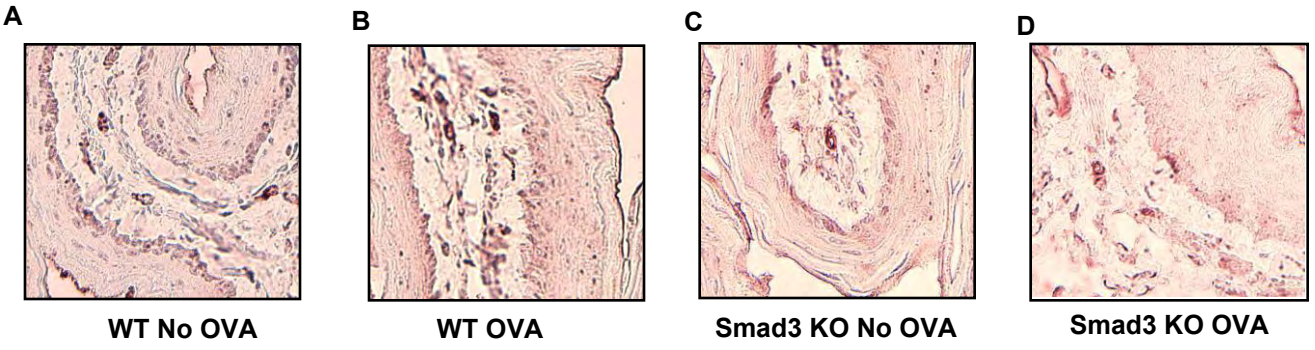


Figure 3

Figure 4



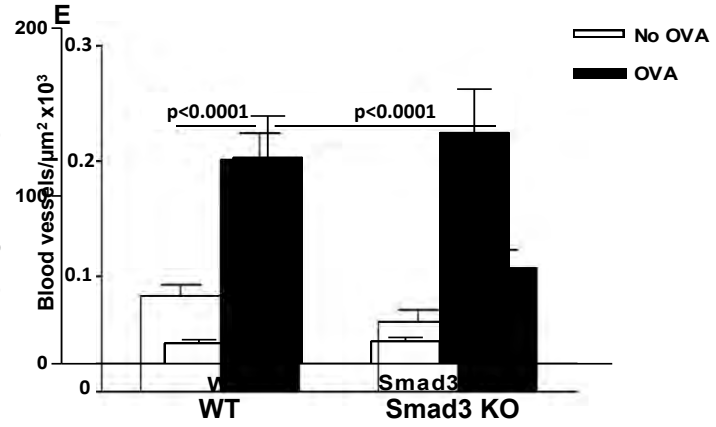
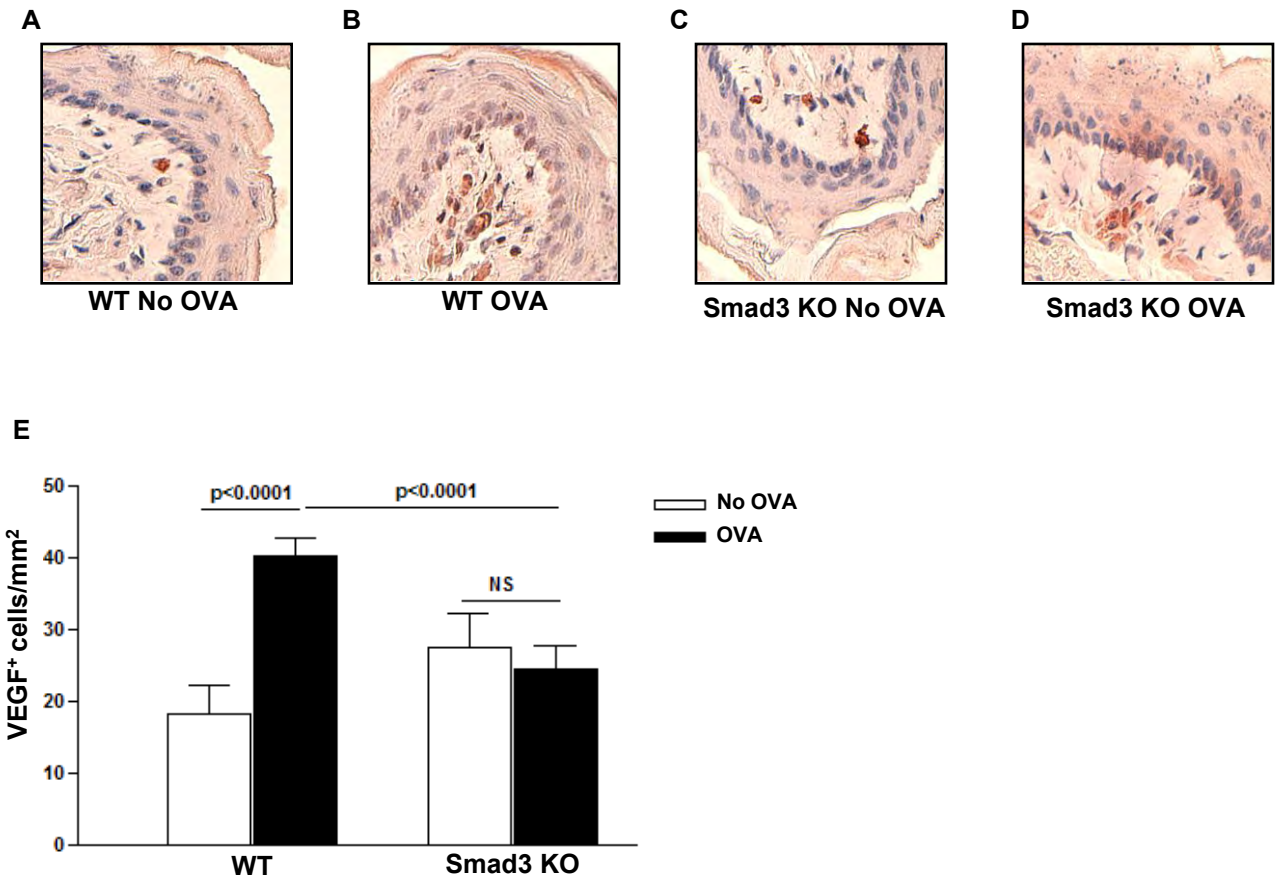


Figure 4

Figure 5



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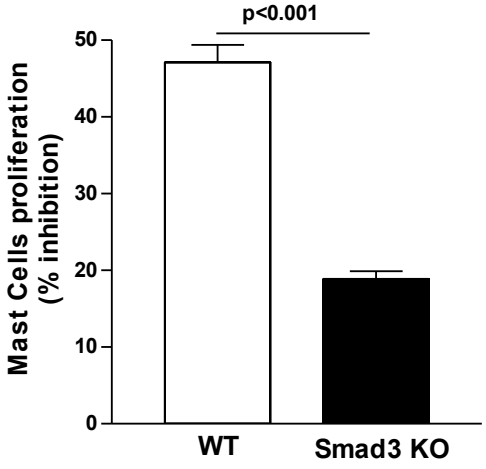
p<0.001
Mast Cells proliferation
(% inhibition)
50
40
30
20
10
0
WT
Smad3 KO

Figure 6

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Targeting AMCase reduces esophageal eosinophilic inflammation and remodeling in a mouse model of egg induced eosinophilic esophagitis ☆

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ABSTRACT

Studies of AMCase inhibition in mouse models of lung eosinophilic inflammation have produced conflicting results with some studies demonstrating inhibition of eosinophilic inflammation and others not. No studies have investigated the role of AMCase inhibition in eosinophilic esophagitis (EoE). We have used a mouse model of egg (OVA) induced EoE to determine whether pharmacologic inhibition of AMCase with allosamidin reduced eosinophilic inflammation and remodeling in the esophagus in EoE. Administration of intra-esophageal OVA for 6 weeks to BALB/c mice induced increased levels of esophageal eosinophils, mast cells, and features of esophageal remodeling (fibrosis, basal zone hyperplasia, deposition of the extracellular matrix protein fibronectin). Administration of intraperitoneal (ip) allosamidin to BALB/c mice significantly inhibited AMCase enzymatic activity in the esophagus. Pharmacologic inhibition of AMCase with ip allosamidin inhibited both OVA induced increases in esophageal eosinophilic inflammation and OVA induced esophageal remodeling (fibrosis, epithelial basal zone hyperplasia, extracellular matrix deposition of fibronectin). This inhibition of eosinophilic inflammation in the esophagus by ip allosamidin was associated with reduced eotaxin-1 expression in the esophagus. Oral allosamidin inhibited eosinophilic inflammation in the epithelium but did not inhibit esophageal remodeling. These studies suggest that pharmacologic inhibition of AMCase results in inhibition of eosinophilic inflammation and remodeling in the esophagus in a mouse model of egg induced EoE partially through effects in the esophagus on reducing chemokines (i.e. eotaxin-1) implicated in the pathogenesis of EoE.

1. Introduction

Chitinases (such as acid mammalian chitinase the focus of this study) are a family of evolutionary conserved enzymes present not only in lower life forms but also in mammals that can cleave chitin a naturally occurring polysaccharide composed of N-acetylglucosamine repeats [1,2]. Chitin is highly expressed in insects and crustacean exoskeletons, fungal cell walls, and microfilarial nematode sheaths [3]. Levels of chitin are regulated by enzymes synthesizing chitin (i.e. chitin synthase) or degrading chitin (i.e. chitinases). Although mammals do not synthesize chitin (as they do not express enzymes such as chitin synthase), they do express enzymes that can degrade chitin such as acid mammalian chitinase (AMCase) [4]. Studies in mice and humans suggest that chitinases may modulate the innate immune response by either interacting with chitin [5,6], or by exerting effects on the immune system independent of actions on chitin [7,8]. AMCase is a member of the mammalian chitinase family that has been studied in the context of eosinophilic inflammation and asthma [2,7,9–13], but not in eosinophilic esophagitis (EoE) which is the focus of this study. In studies of mouse models of asthma, expression of AMCase can be induced in the lung by IL-13 [7] and allergens [10–13]. IL-13 transgenic mice have increased AMCase expression in the lung (in macrophages and epithelial cells), associated with increased eosinophilic lung inflammation [7]. Administration of a pharmacologic inhibitor of AMCase (allosamidin) to IL-13 transgenic mice inhibited AMCase activity and also inhibited eosinophilic lung inflammation [7] suggesting an important role for AMCase in mediating the effect of IL-13 in inducing eosinophilic lung inflammation. As IL-13 regulates AMCase expression [7], is highly expressed in the esophagus in eosinophilic esophagitis (EoE) [14], and the IL-13 transcriptome is implicated in the pathogenesis of EoE [14], in this study we have used a mouse model of egg (OVA) induced EoE to examine whether AMCase is expressed in the esophagus and whether blocking AMCase activity with a pharmacologic inhibitor

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inhibits esophageal eosinophilic inflammation and remodeling of the esophagus.

EoE is a disease defined by combined clinical and histological criteria (>15 esophageal eosinophils/hpf) [15]. Increased numbers of mast cells are also noted in the esophagus in EoE [16]. In addition to the presence of inflammation of the esophagus, EoE may be complicated by the development of remodeling of the esophagus [17] and stricture formation which may necessitate repeated esophageal dilation to prevent food impactions [15]. Features of esophageal remodeling in EoE include esophageal fibrosis, epithelial basal zone thickening, and angiogenesis [15–17]. Both eosinophils and mast cells likely contribute to esophageal remodeling in EoE as increased numbers of eosinophils expressing the pro-remodeling cytokine TGF-β1 are present in the remodeled esophagus in EoE [15–17]. In addition to increased eosinophils in EoE, increased numbers of tryptase positive mast cells are present in the esophageal smooth muscle in EoE, express TGF-β1, and increase the contractility of human esophageal smooth muscle cells in vitro [16]. As such, mast cells localized to the smooth muscle in patients with EoE might modulate esophageal contractility and contribute to symptoms in EoE.

As EoE is associated with significant symptoms (nausea, vomiting, abdominal pain) and complications from remodeling such as stricture formation and food impaction, therapeutic options are needed that reduce not only esophageal eosinophilic inflammation, but also mast cells, and remodeling. In this regard current therapeutic options include elemental diet which eliminates symptoms and underscores the food dependence of EoE [15]. However, difficulties with diet adherence result in this not readily being acceptable as a long term option for most patients with EoE. Topical corticosteroids reduce esophageal eosinophils, mast cells, and features of remodeling in many but not all subjects with EoE [18]. Novel therapies such as anti-IL-5 significantly reduce eosinophilic inflammation but have not reduced symptoms in studies of EoE [19]. In this study we have used a mouse model of food (i.e. egg) induced EoE previously described in this laboratory [20] to demonstrate that allosamidin a pharmacologic inhibitor of AMCase [21] inhibits AMCase activity in the esophagus and reduces esophageal levels of eosinophils, and features of esophageal remodeling suggesting that allosamidin may be a novel therapy to investigate in EoE.

2. Material and methods

2.1. Oral egg (OVA) allergen induced eosinophilic esophagitis

Eight- to ten week-old female BALB/c mice (12 mice/group; Charles River Labs Inc., Wilmington, MA) were sensitized intraperitoneally with 50 μg OVA adsorbed to 1 mg of aluminum hydroxide adjuvant (OVA; grade V: Sigma-Aldrich, St. Louis, MO) on day 0 and day 14 and then challenged intra-esophageally three times/week for four weeks with 10 mg OVA suspended in 100 μl PBS on days 28, 30, 32, 35, 37, 39, 42, 44, 46, 49, 51, and 53 as previously described in this laboratory [20]. OVA was administered through an intragastric feeding needle (20-gauge, 1.5-inch; Pepper and Sons, Inc., New Hyde Park, NY). Mice were sacrificed 24 h after the last administration of intra-esophageal OVA (day 54). Control BALB/c mice were neither sensitized nor challenged. The esophagus was removed in its entirety and fixed with 4% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA) for 24 h., oriented and embedded in 1% agarose (Invitrogen, Carlsbad, CA), and subsequently 5 μm sections (upper, middle and lower) embedded in paraffin. Esophageal sections from each layer were included for analysis of esophageal inflammation and esophageal remodeling. In each experiment in which stained and immunostained slides were quantified by image analysis, identical light microscope conditions, magnification, gain, camera position, and background illumination were utilized. The analysis of slides was performed by an investigator blinded to the study group. All animal experimental protocols were approved by the University of California, San Diego Animal Subjects Committee.

2.2. Pharmacologic intervention with chitinase inhibitor allosamidin in mouse model of OVA induced eosinophilic esophagitis

Three different groups of BALB/c mice (n = 12 mice/group) were studied: Group 1, no OVA; Group 2, OVA + chitinase inhibitor allosamidin [21]; and Group 3, OVA + control diluent. Allosamidin (1 mg/kg, a kind gift from Dr. Sakuda, Tokyo University, Japan) or PBS diluent was administered in a volume of 100 μl intraperitoneally 1 h before each of the twelve intra-esophageal OVA challenges (administered between days 28 and 53) as described above. In selected experiments allosamidin (1 mg/kg) or PBS diluent was administered using an intragastric feeding needle (as described above for OVA challenges) in a volume of 100 μl intra-esophageally 1 h before each of the twelve intra-esophageal OVA challenges.

2.3. Eosinophil quantitation in esophagus

Eosinophils were detected in esophageal tissue by immunohistochemistry using an anti-mouse major basic protein (MBP) antibody (kindly provided by James Lee PhD, Mayo Clinic, Scottsdale, AZ). Quantitation of the number of eosinophils was performed using a light microscope attached to an image-analysis system with the entire cross section of the esophagus visualized [20]. The area of the esophageal lamina propria, epithelium, or esophageal muscle layer was outlined and this area was determined by the image analysis software (Image-Pro Plus; Media Cybernetics). Results are expressed as either the number of eosinophils per mm^2 of lamina propria, the number of eosinophils per mm^2 of esophageal epithelium, or the number of eosinophils per mm^2 of esophageal muscle layer.

2.4. Mast cell quantitation in esophagus

As increased esophageal mast cells are also a prominent feature of EoE [16], esophageal sections were also stained for mast cells using chloroacetate esterase as previously described in this laboratory [22]. Results are expressed as the number of mast cells per mm^2 of lamina propria.

2.5. Quantitation of esophageal remodeling

We quantitated features of esophageal remodeling characteristic of EoE including esophageal fibrosis, epithelial basal zone hyperplasia, and extracellular deposition of matrix proteins [17,20].

2.5.1. Esophageal trichrome staining

The area of esophageal trichrome staining (as an index of collagen deposition) in paraffin embedded esophagus was outlined and quantified using a light microscope (Leica DMLS, Leica Microsystems Inc., NY) attached to an image analysis system (Image-Pro Plus, Media Cybernetics, MI) as previously described [17,20]. Results are expressed as the area in $μm^2$ of trichrome staining per $μm^2$ total area of lamina propria.

2.5.2. Esophageal collagen

The amount of esophageal collagen was measured with a collagen assay that uses a dye reagent that selectively binds to the [Gly-X-Y]n tripeptide sequence of mammalian collagens (Biocolor) as previously described in this laboratory [23]. In all experiments, a collagen standard was used to calibrate the assay. Results are expressed as μg collagen.

2.5.3. Deposition of extracellular matrix protein

The deposition of the extracellular matrix protein fibronectin is associated with tissue remodeling [22]. We used two methods (ELISA and

immunohistochemistry) to quantitate fibronectin deposition in the esophagus. Levels of fibronectin in esophagus lysates were assayed by ELISA (Assaypro, St. Charles, MO). The sensitivity of the fibronectin assay is 50 ng/ml. Esophageal lysates were prepared by homogenizing esophageal tissue in lysis buffer as previously described in this laboratory [23]. Esophageal supernatants (obtained by centrifugation of lysates at 10,000 *g* for 20 min) were passaged through 0.8 μm pore size filter and frozen at −80 °C in polypropylene tubes until used in ELISA assays.

The area of fibronectin immunostaining in the esophagus was quantitated by immunohistochemistry using an anti-fibronectin Ab (Abcam, Cambridge, MA) and quantified using a light microscope attached to an image analysis system. Results are expressed as the area of fibronectin staining per total esophageal LP area.

2.5.4. Esophageal epithelial basal zone thickness

Basal zone hyperplasia is a feature of EoE [17,20]. The epithelial basal zone thickness was quantitated in esophageal sections stained with hematoxylin and eosin using a light microscope attached to an image-analysis system. The thickness of the basal layer was recorded in μm in four randomly selected areas of esophageal epithelium in each slide.

2.5.5. Esophageal TGF-β1 + cells

Esophageal tissue sections were processed for immunohistochemistry using a primary mAb directed against either TGF-β1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as described [17,20]. Results are expressed as the number of TGF-β1 positive cells per mm^2 of lamina propria.

2.6. Esophageal levels of chemokines/cytokines that regulate eosinophilic inflammation

Levels of the eosinophil chemoattractant eotaxin-1, and Th2 cytokines (IL-5, IL-13) were measured in esophageal lysates by ELISA (R&D System Inc., Minneapolis, Minn). The IL-5, IL-13, and eotaxin-1 assays each had a sensitivity of 15 pg/ml.

2.7. Chitinase bioactivity assay

Levels of AMCase enzymatic activity was measured in the homogenized esophageal lysates of mice treated with either allosamidin or diluent control. AMCase enzyme activity was determined using the AMCase fluorogenic substrate 4MU-chitobiose (4-methyllumbelliferyl-β-D-N,N′-diacetylchitobiose; Sigma, St. Louis, MO) [4] in an acidic environment (pH ~ 4.5) at 37 °C. The florescence of liberated 4MU (4-methyllumbelliferone) was measured using a fluorimeter with excitation at 360 nm and emission at 450 nm [4].

2.8. Statistical analysis

Results were compared by a Mann–Whitney test using a statistical software package (GraphPad Prism, San Diego, CA). p values <0.05 were considered statistically significant. Results are presented as mean ± SEM.

3. Results

3.1. Allosamidin ip inhibits oral OVA induced eosinophilic inflammation in the esophagus

Oral OVA challenge induced a significant increase in esophageal eosinophils (Fig. 1A–B). Eosinophils were infiltrated into the esophageal epithelium, lamina propria, and muscle layer. OVA challenge increased the total number of MBP + eosinophils in the esophagus including the epithelium (OVA vs no OVA) ($p = 0.0001$) (Fig. 1C), the lamina propria (OVA vs no OVA) ($p = 0.0001$) (Fig. 1D), and the muscle layer (OVA vs no OVA) ($p = 0.0001$) (Fig. 1E).

IP administration of the AMCase inhibitor allosamidin induced a significant decrease in esophageal eosinophils (Fig. 1). Allosamidin ip induced a 63% decrease in eosinophil numbers in esophageal epithelium (OVA vs allosamidin ip + OVA) ($p = 0.03$) (Fig. 1C), a 50% decrease in eosinophils in the lamina propria (OVA vs allosamidin ip + OVA) ($p = 0.04$) (Fig. 1D), and a statistically insignificant trend of fewer in eosinophils in the muscle layer (OVA vs allosamidin ip + OVA) ($p = 0.8$) (Fig. 1E).

3.2. Allosamidin ip inhibits oral OVA induced esophageal remodeling

3.2.1. Esophageal fibrosis

Oral OVA challenge induced a significant increase in esophageal fibrosis as assessed by either the area of esophageal trichrome staining (OVA vs no OVA) ($p = 0.0001$) (Fig. 2A) or the amount of esophageal collagen (OVA vs no OVA) ($p = 0.001$) (Fig. 2B). Allosamidin ip decreased the area of esophageal trichrome staining by 18% (OVA vs allosamidin ip + OVA) ($p = 0.004$) (Fig. 2A). Although allosamidin ip also decreased the amount of esophageal collagen by a similar amount to that detected with trichrome staining (i.e. 18%), this decrease in collagen approached but did not reach statistical significance (OVA vs allosamidin ip + OVA) ($p = 0.08$) (Fig. 2B).

3.2.2. Esophageal deposition of fibronectin

Levels of deposition of the extracellular matrix protein fibronectin were significantly increased in the esophagus following oral OVA challenge as assessed by either the area of esophageal fibronectin staining (OVA vs no OVA) ($p = 0.0001$) (Fig. 2C) or the amount of esophageal fibronectin as quantitated by ELISA (OVA vs no OVA) ($p = 0.02$) (Fig. 2D). Allosamidin ip decreased the area of esophageal fibronectin staining in oral OVA challenged mice (OVA vs allosamidin ip + OVA) ($p = 0.0001$) (Fig. 2C), and the amount of esophageal fibronectin quantitated by ELISA (OVA vs allosamidin ip + OVA) ($p = 0.002$) (Fig. 2D).

3.2.3. Esophageal epithelial basal zone hyperplasia

The thickness of the esophageal epithelial basal zone was significantly increased following OVA challenge (OVA vs no OVA) ($p = 0.0001$) (Fig. 2E). Allosamidin ip decreased the thickness of the esophageal epithelial basal zone (OVA vs allosamidin ip + OVA) ($p < 0.0001$) (Fig. 2E).

3.2.4. Esophageal TGF-β1 + cells

The number of esophageal TGF-β1+ cells increased significantly in OVA challenged mice ($p = 0.01$) (Fig. 2F). Allosamidin ip decreased the number of esophageal TGF-β1+ cells (OVA vs allosamidin ip + OVA) ($p = 0.05$) (Fig. 2F).

3.3. Allosamidin ip inhibits oral OVA induced expression of eotaxin-1 in the esophagus

Oral OVA challenge induced a significant increase in esophageal eotaxin-1 (OVA vs no OVA) ($p = 0.01$) (Fig. 3A), IL-13 (OVA vs no OVA) ($p = 0.004$) (Fig. 3B), but not IL-5 (OVA vs no OVA) (p = ns) (Fig. 3C) as assessed by ELISA. Administration of allosamidin ip to oral OVA challenged mice significantly reduced levels of esophageal eotaxin-1 (OVA vs allosamidin ip + OVA) ($p = 0.05$) (Fig. 3A). Allosamidin ip induced a trend for reduction in IL-13 which was not significant (OVA vs allosamidin ip + OVA) ($p = 0.11$) (Fig. 3B), and no reduction in IL-5 (OVA vs allosamidin ip + OVA) (p = ns) (Fig. 3C).

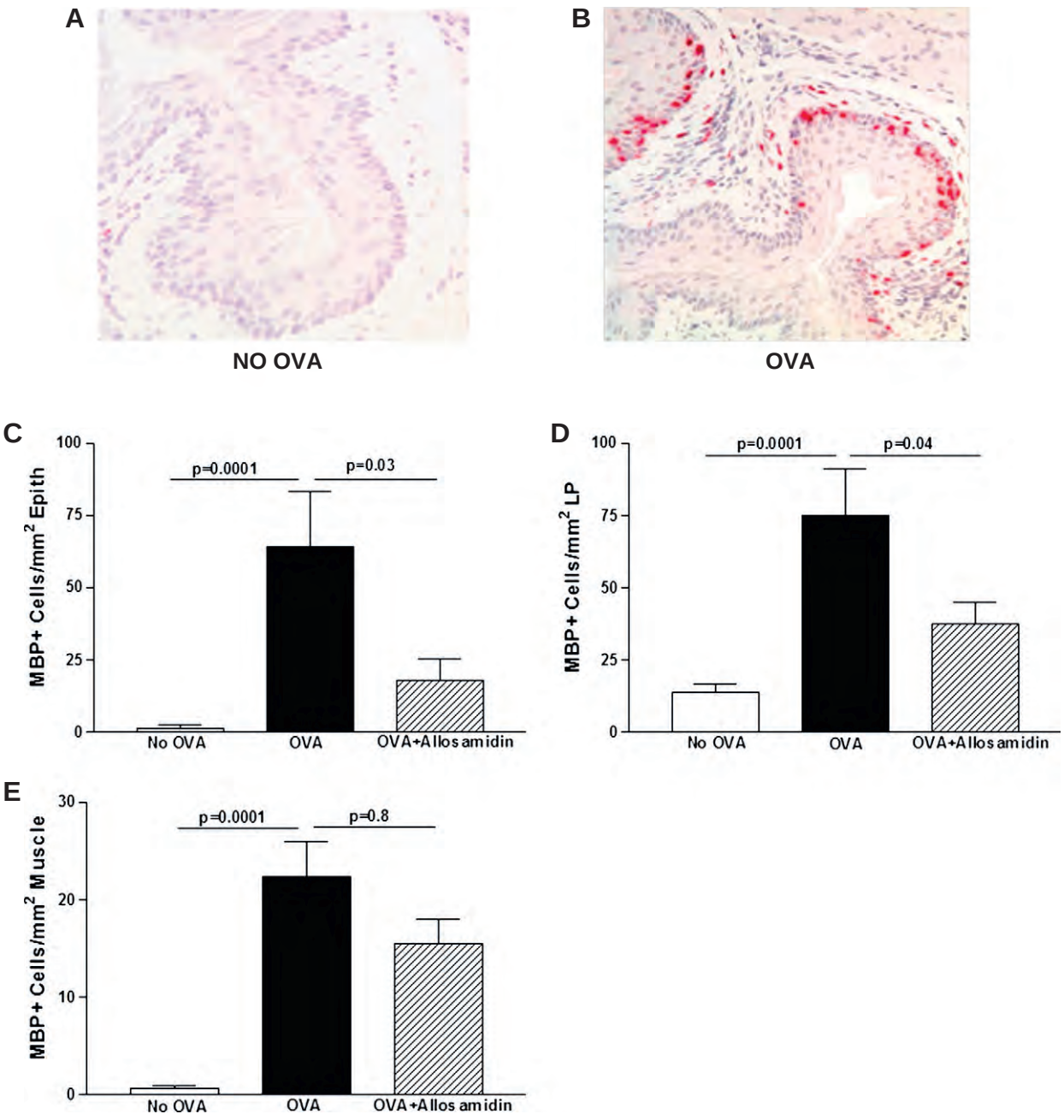

Fig. 1. Effect of allosamidin on oral OVA induced eosinophilic inflammation in esophagus. The esophagus from non-OVA challenged (A) and OVA challenged (B) mice were immunostained with an anti-MBP Ab. The number of MBP + eosinophils in the esophagus was quantitated per mm^2 of epithelium (Fig. 1C), per mm^2 of lamina propria (Fig. 1D), and per mm^2 of muscle layer (Fig. 1E) in three different groups (no OVA; OVA + diluent; OVA + allosamidin) (n = 12 mice/group).

3.4. Effect of allosamidin ip on oral OVA induced mast cell inflammation in the esophagus

Oral OVA challenge induced a significant increase in mast cells in the lamina propria ($p = 0.001$) (Fig. 4), but not the epithelium (data not shown). Administration of allosamidin ip to oral OVA challenged mice resulted in a trend for reduced numbers of mast cells that approached but did not reach statistical significance (OVA vs allosamidin ip + OVA) ($p = 0.07$) (Fig. 4).

3.5. Effect of allosamidin ip on AMCase activity in the esophagus

The esophagus from non-OVA challenged mice expressed constitutive AMCase enzymatic activity (Fig. 5). Following oral OVA challenge there was a slight but statistically significant increase in AMCase enzymatic activity in the esophagus (OVA vs no OVA) ($p = 0.03$) (Fig. 5). Administration of allosamidin ip significantly reduced AMCase activity in the esophagus ($p = 0.001$) (Fig. 5).

3.6. Effect of oral allosamidin on OVA induced esophageal eosinophilic inflammation

Oral administration of allosamidin significantly reduced the number of eosinophils in the esophageal epithelium of OVA challenged mice (OVA vs oral allosamidin + OVA) ($p = 0.03$) (Fig. 6A). While there was a trend for oral allosamidin to decrease the number of eosinophils in the lamina propria (Fig. 6B), and smooth muscle (Fig. 6C) this was not statistically significant. Oral administration of allosamidin did not inhibit basal layer thickness, or esophageal fibrosis as assessed by the area of trichrome staining (data not shown).

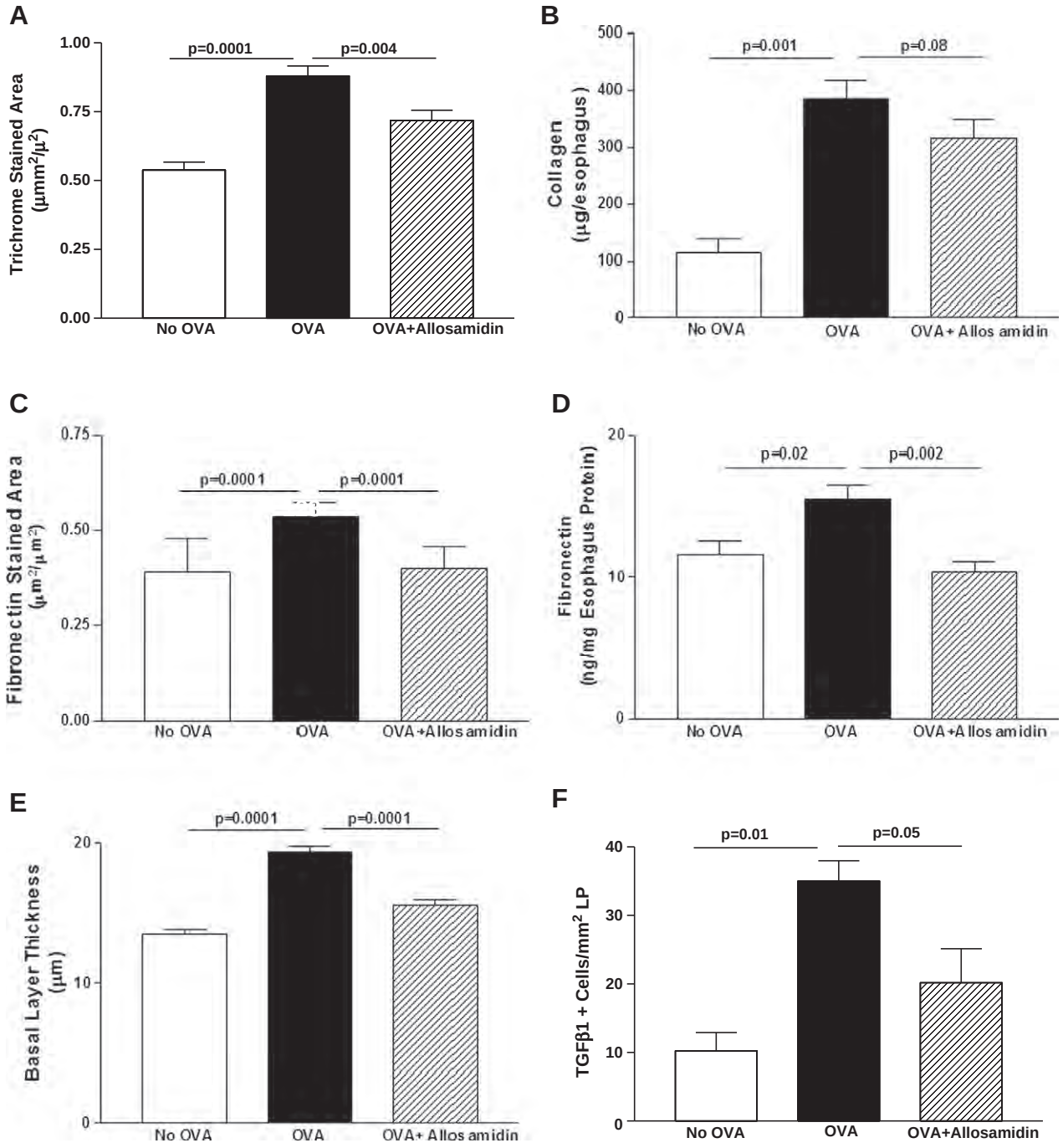

Fig. 2. Effect of allosamidin on oral OVA induced esophageal remodeling. The esophagus from mice belonging to three different groups (no OVA; OVA + diluent; OVA + allosamidin) (n = 12 mice/group) was processed to detect features of esophageal remodeling including, the area of esophageal trichrome staining (A), the amount of esophageal collagen (B), the area of esophageal fibronectin staining (C), the amount of esophageal fibronectin quantitated by Elisa (D), the thickness of the esophageal epithelial basal zone layer (E), and the number of esophageal TGFβ1 positive cells (F).

4. Discussion

In this study we have demonstrated in a mouse model of egg induced EoE that administration of a pharmacologic inhibitor of AMCase (i.e. allosamidin) ip significantly inhibited levels of esophageal AMCase activity, esophageal eosinophilic inflammation, as well as levels of esophageal remodeling including fibrosis, epithelial basal zone hyperplasia, and deposition of extracellular matrix proteins. The mechanism by which ip allosamidin inhibits esophageal eosinophilic inflammation involves effects in the esophagus to reduce key chemokines (i.e. eotaxin-1) regulating esophageal eosinophilic inflammation. Although previous studies of ip allosamidin in mouse models of asthma have demonstrated that it can inhibit eosinophilic lung inflammation [7,10,11], this is the first study to demonstrate that ip allosamidin can inhibit eosinophilic inflammation in the esophagus and describe potential mechanisms mediating this effect. For example, we have made the novel observation that ip allosamidin exerts inhibitory effects on key eosinophil chemokines expressed in the esophagus. Moreover, this is the first study to demonstrate that ip allosamidin can inhibit tissue remodeling which in EoE is important to the subset of subjects who develop esophageal strictures and food impactions. As eosinophils express TGF-β1 [17], the ability of allosamidin to inhibit the

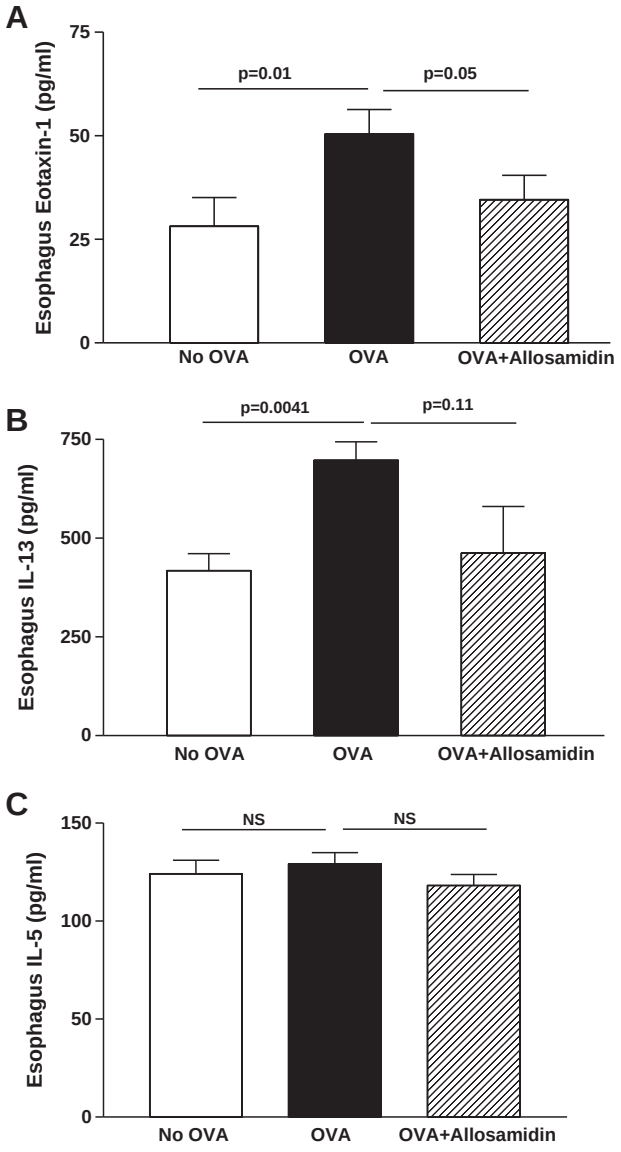

Fig. 3. Effect of allosamidin on oral OVA induced esophageal levels of IL-13, eotaxin-1, and IL-5. The esophagus from mice belonging to three different groups (no OVA; OVA + diluent; OVA + allosamidin) (n = 12 mice/group) was processed for ELISA to quantitate levels of eotaxin-1 (A), IL-13 (B), and IL-5 (C).

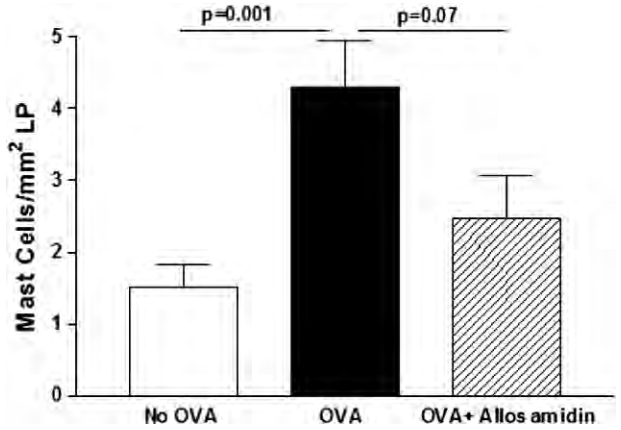

Fig. 4. Effect of allosamidin on oral OVA induced mast cell inflammation in esophagus. The esophagus from mice belonging to three different groups (no OVA; OVA + diluent; OVA + allosamidin) (n = 12 mice/group) was stained with chloroacetate esterase to detect mast cells which were quantitated per mm^2 of lamina propria.

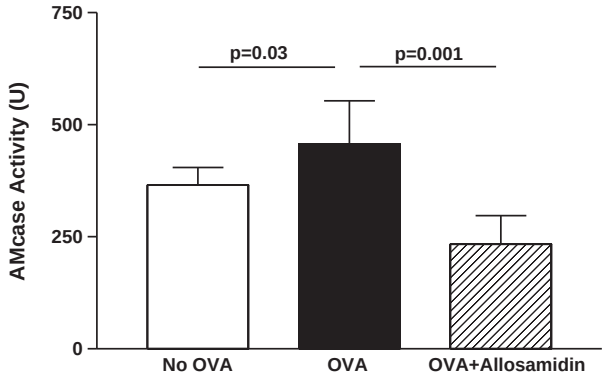

Fig. 5. Effect of allosamidin on oral OVA induced AMCase enzymatic activity in esophagus. The esophagus from mice belonging to three different groups (no OVA; OVA + diluent; OVA + allosamidin) (n = 12 mice/group) was processed to detect AMCase enzymatic activity using the substrate 4MU-chitobiose.

accumulation of eosinophils in the esophagus may result in reduced levels of TGF-β1 expression with resultant reduced levels of esophageal remodeling.

Insight into the mechanism by which allosamidin inhibits eosinophilic inflammation in the esophagus may be derived from studies of allosamidin, and its target AMCase. Allosamidin is a pseudotrisaccharide consisting of two *N*-acetyl-D-allosamine sugars, linked to a novel moiety termed allosamizoline [21,24]. It was originally isolated from *Streptomyces* cultures [24] and has also been totally synthesized [25]. On the basis of sequence homologies, chitinases belong to a subset of the large family of glycosyl hydrolases (family 18 of which AMCase is a member; and family 19) [26]. Members of family 18 such as AMCase employ a substrate-assisted reaction mechanism [27], whereas those of family 19 adopt a fold-and-reaction mechanism similar to that of lysozyme [28], suggesting that these families of chitinases evolved independently to deal with chitin. Allosamidin inhibits all family 18 chitinases including AMCase (but not family 19 chitinases), with K_i in the nm to μm range [25]. The crystal structure of allosamidin complexed with human chitinase has also been described demonstrating that allosamidin binds in a groove on the chitinase [29].

Two mammalian chitinases have been cloned (AMCase and chitotriosidase) [4,30]. AMCase has been implicated in the pathogenesis of asthma [7,10,11], while increased expression of chitotriosidase is observed in lysosomal lipid storage disorders like Gaucher disease [31]. AMCase is an enzyme characterized by an acidic isoelectric point and therefore named acidic mammalian chitinase [4]. The enzyme is extremely acid stable and its constitutive expression is relatively abundant in the gastrointestinal tract and to a lesser extent in the lung [32]. AMCase is synthesized as a 50-kDa protein containing a 39-kDa N-terminal catalytic domain, a hinge region, and a C-terminal chitin-binding domain [4]. AMCase is expressed in alveolar macrophages and in the gastrointestinal tract where it has been hypothesized to play a role in digestion and/or host defense [4,32].

Studies in mouse models of asthma have provided conflicting evidence as to whether targeting AMCase inhibits eosinophilic lung inflammation [7,10,11], or not [12,13]. The studies in mouse models of asthma demonstrating that targeting AMCase inhibits eosinophilic lung inflammation include studies using pharmacologic inhibitors of AMCase (i.e. ip allosamidin) [7,10] and siRNA approaches to knockdown AMCase [11], while the studies that have not shown an inhibitory effect of targeting AMCse have also used pharmacologic inhibitors of AMCase (i.e. ip allosamidin) [12], and AMCase mutant mice [12,13]. It is unclear why these studies have resulted in differing results. Potential explanations include difference in protocols including the nature of the allergens used (fungal allergen *Aspergillus* which contains high levels of chitin vs OVA or dust mite), the allergen challenge protocol used, potential

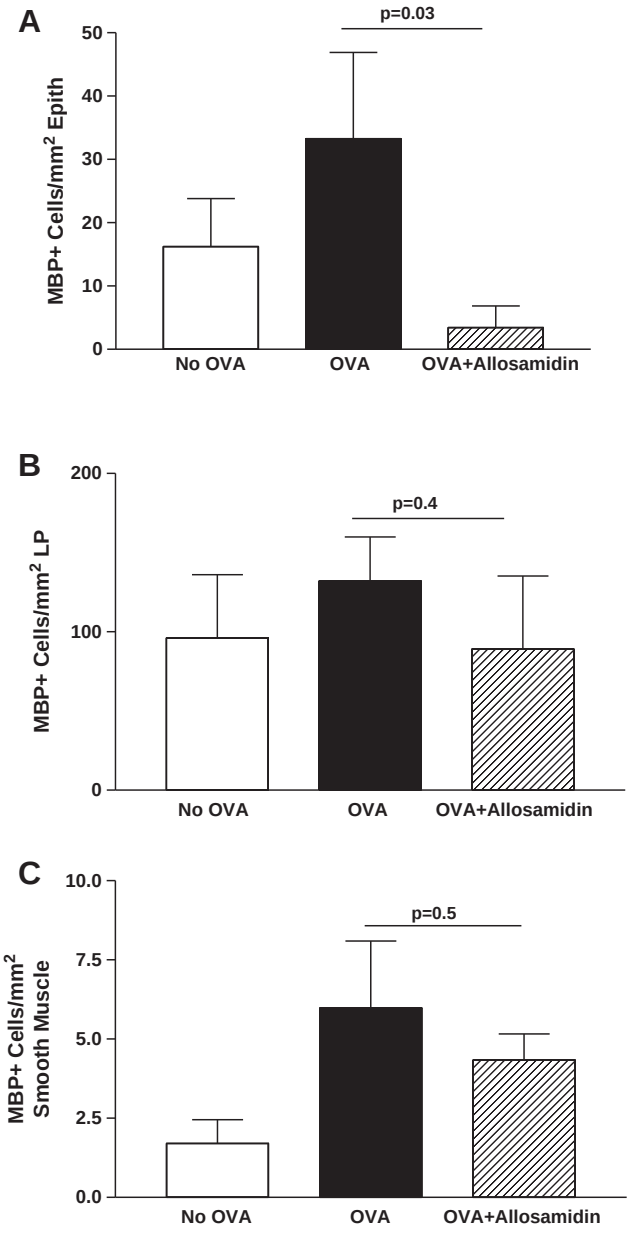

Fig. 6. Effect of oral allosamidin on oral OVA induced eosinophilic inflammation in esophagus. The number of MBP + eosinophils in the esophagus was quantitated per mm^2 of epithelium (A), per mm^2 of lamina propria (B), and per mm^2 of muscle layer (C) in three different groups (no OVA; OVA + diluent; OVA + oral allosamidin) (n = 5 mice/group).

off target effects of pharmacologic inhibitors, and potential upregulation of compensatory pathways in studies using AMCase mutant mice.

We also performed studies to determine whether orally administered allosamidin could inhibit eosinophilic inflammation in the esophagus. As all prior in vivo studies with allosamidin in mice have used ip administration, we were unable to be guided by prior studies as to what might be an optimal oral allosamidin dose and dosing schedule. We selected an oral dose of allosamidin (1 mg/kg) that was the same as the ip dose used in this and other studies [7,21]. This oral dose of allosamidin significantly inhibited esophageal epithelial eosinophilic inflammation. However, this oral dose of allosamidin was not effective in inhibiting eosinophilic inflammation in the LP, or in inhibiting esophageal fibrosis. Future studies in which the daily administration of oral allosamidin (only administered once prior to each OVA challenge in this study), and the dose of allosamidin is varied, will define the maximal inhibitory effect of oral allosamidin in this mouse model of EoE.

In summary, our study is the first study examining the effect of using ip allosamidin to inhibit AMCase and reduce eosinophilic inflammation in an organ other than the lung. Using a mouse model of egg induced EoE we have demonstrated that ip allosamidin significantly inhibited eosinophilic inflammation in the esophageal epithelium and lamina propria, but did not inhibit eosinophilic inflammation in smooth muscle. The reduction in esophageal expression of eotaxin-1 in allosamidin treated mice may be one mechanism by which allosamidin reduces esophageal eosinophilic inflammation. Furthermore, we demonstrate that ip allosamidin significantly reduces esophageal remodeling (fibrosis, extracellular deposition of extracellular matrix protein fibronectin, basal zone hyperplasia) a key finding in subjects with EoE and esophageal stricture formation [16,17]. Thus, targeting AMCase may be a novel therapeutic option in EoE, especially in the subset of EoE subjects with esophageal remodeling who may be at higher risk for esophageal stricture formation and food impactions.

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